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Synthèse et évaluation des activités antimicrobiennes de nouveaux dérivés 3,20-bis(polyaminostéroïdiens). Applications en thérapeutique humaine

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Sommaire

Avant propos :

Le format de présentation de cette thèse correspond à une recommandation de la spécialité des Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier Raoult

Résumé

Les dérivés aminostéroïdiens analogues de la squalamine ont été largement étudiés pour leur large spectre d'activité sur les bactéries et les champignons multirésistants. Dans ce travail, nous avons réalisé la synthèse de nouveaux dérivés aminostéroïdiens et présentant de nombreuses charges positives liées à la présence de groupements azotés, les 3,20-bis(polyaminostéroïdes) analogues de la squalamine. Une étude de la relation structure-activité a démontré l'importance des charges positives induites par la présence d'atomes d'azote dans les chaines carbonées portées par le motif cholestane. Nous avons également pu mettre en évidence les mécanismes d'action mis en œuvre vis-à-vis des bactéries Gram positive et Gram négative. L'étude des activités antifongiques démontre que la squalamine et le dérivé aminostéroïdien DAS-1 possèdent de bonnes activités sur diverses souches de levures impliquées dans de nombreuses fongémies, avec des CMIs variant de 1 à 16 µg/mL. Par ailleurs, nous avons étudié les applications potentielles de ces dérivés et ainsi pu tout d'abord démontrer que ces dérivés en formulation de pommades à 1% de squalamine et de dérivé 3.20-bis(polyaminostéroïdien) étaient capables de réduire efficacement la colonisation cutanée à *S. aureus* sur un modèle animal. Il a été démontré que ces dérivés bis(polyaminostéroïdiens) sont également très actifs vis-à-vis de bactérie et de champignons multirésistants isolés des patients mucoviscidosiques. Nous avons également testé leur activité en tant qu'agent désinfectant de matériel médical et plus particulièrement les nébuliseurs. La formation de cachets hydrosolubles à base de squalamine, nous a ainsi permis de développer avec succès un modèle de désinfection simple, rapide et peu onéreux des nébuliseurs contaminés.

Mots clés : Squalamine, dérivés 3,20-bis(polyaminostéroïdiens), désinfection, décolonisation.

Summary

Aminosterol derivatives analogues of squalamine possess a broad spectrum activity against multidrug resistant bacteria and fungi. We synthesized a new series of 3,20-bis(polyaminosteroïd) analogues involving a titanium reductive amination possessing numerous positives charges due to the presence of nitrogen groups. The study of relation structure-activity demonstrates that the nature of the amino group attached to the sterol plays a crucial role on antimicrobial activity of these compounds. We had also determined the mechanism of action of bis(polyaminosteroïd) on Gram negative and Gram positive bacteria. The study of antifungal activity of squalamine and aminosterol derivative ASD-1 show a good activity against various yeast responsible of fungal infections, minimal inhibitrice concentration ranging from 1 to 16 µg/mL. We studied a potential application of these compounds in human therapeutic. We evaluated squalamine and related parent-derived ointments (1%) as potential new compounds for *S. aureus* decolonization in a new mouse model. Using this model we found that squalamine ointment (1%) was able to reduce efficiently *S. aureus* colonization. Squalamine and bis(polyaminosteroïd) derivatives were actives against multidrug resistant bacteria and fungi isolated from cystic fibrosis patients. We investigated the potential use of squalamine compound *in vitro* in a nebulizer disinfection model. A formulation of squalamine disinfecting soluble tablets at 2.5 % (W/W) was developed and successfully applied for rapid nebulizer disinfection.

Keywords : Squalamine, 3.20-bis(polyaminosteroïds) derivatives, disinfection, decolonization.

Introduction

Les antibiotiques ont été largement utilisés, au cours de cette dernière décennie, pour traiter diverses maladies infectieuses qui restent une des causes prédominantes de mortalité et de morbidité dans le monde. Néanmoins, l'utilisation massive de ces antibiotiques a conduit à l'émergence de pathogènes multirésistants aux antibiotiques classiques[1]. Parmi ces pathogènes résistants, on peut citer le cas de *Staphylococcus aureus* résistant à la méthicilline, Enterococcus résistant à la vancomycine et *Klebsiella pneumoniae NDM-1* qui posent les limites des traitements thérapeutiques utilisés à l'heure actuelle. Une des stratégies possibles pour limiter l'apparition du phénomène de résistance aux antibiotiques est la surveillance de leur prescription mais également le développement de nouvelles molécules [2]. Les polyamines ont été décrites comme des composés comportant diverses charges positives dues à la présence de groupements aminés chargés positivement [3-4]. Récemment de nombreuses activités ont été rapportées pour des polyamines telles que des activités antimicrobiennes et antivirales [5-6] et les polyaminostérols ont suscité particulièrement l'intérêt de nombreux chercheurs [7]. Parmi ces composés, la squalamine et la trodusquemine, deux stéroïdes cationiques naturels, ont été isolés à partir d'un requin nommé *Squalus acanthias* en 1993 principalement dans le foie et la vésicule biliaire du requin [8]. La squalamine et la trodusquemine présentent une

structure similaire de type cholestane comportant une chaine polyaminée en position 3, une fonction hydroxyle en position 7 et un groupement sulfate en position 24 et ne diffère que par la longueur de la chaine polyaminée. La squalamine a été particulièrement étudiée pour ces propriétés antiangiogeniques et utilisée jusqu'en phase III dans le traitement en association avec d'autres agents anticancéreux des tumeurs malignes [9].

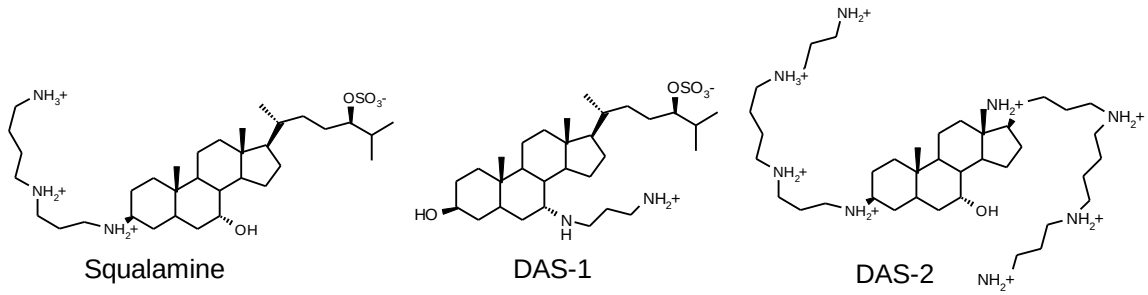

Figure 1. Structure de la squalamine et des dérivés polyaminostéroidiens.

La squalamine présente également des activités antimicrobiennes *in vitro* sur les bactéries Gram positive et Gram négative même sur les souches cliniques multirésistantes. Les activités antimicrobiennes ont été évaluées par la détermination de la concentration minimale d'inhibition (CMIs), les CMIs varient ainsi de 2 à 64 µg/mL sur 137 souches bactériennes différentes [10]. La squalamine présente ainsi deux mécanismes d'actions différents vis-à-vis des bactéries Gram positive et les bactéries Gram négative. Ainsi la squalamine agit par un phénomène de dépolarisation membranaire sur les bactéries Gram positive mesuré par l'efflux d'ATP intracellulaire qui est un indicateur de l'intégrité membranaire avec un efflux maximal d'ATP en moins

de 3 min. La microscopie électronique révèle une libération du contenu intracellulaire observé chez *S. aureus.* Dans le cas des bactéries Gram négative, la membrane externe constitue une barrière pour de nombreuses molécules hydrophobes. Contrairement aux bactéries Gram positive, chez les bactéries Gram négative, nous avons pu démontrer que le relargage d'ATP s'effectue de manière progressive atteignant 35 % d'efflux après 20 min d'incubation. Nous avons pu démontrer que la cible commune de la squalamine et la colistine est la membrane bactérienne, ces deux composés interagissant avec les charges négatives des phosphates de la membrane externe des bactéries Gram négative. La microscopie électronique révèle également la formation de projection en forme de blebs chez *P. aeruginosa* pour la colistine alors que la squalamine démontre un réarrangement du LPS en surface sous forme de micelle. Il a également été démontré que l'addition de cations divalents tels que Ca^{2+} et Mg^{2+} était capable d'inhiber l'activité antimicrobienne de la squalamine contre les bactéries Gram négative alors que l'activité est préservée dans le cas des bactéries Gram positive tel que *S. aureus* [11]. D'autre part, il a été rapporté que la squalamine est active sur de nombreux champignons filamenteux pathogènes résistants aux antifongiques classiques, par un mécanisme d'action qui reste encore mal élucidé, les CMIs variant de 8 à 16 µg/mL sur 52 souches cliniques de champignons issues de patients mucoviscidosiques [12]. Du fait de la difficulté d'obtention de la squalamine qui est présente chez le requin à de très faibles concentrations, différentes voies de

synthèse chimique de cette molécule ont été envisagées. Moriarty a réalisé la première synthèse totale en 17 étapes en 1994 en utilisant l'acide 3 acétoxy-5-cholénique comme substrat de départ [13]. De nombreux dérivés aminostéroïdiens analogues de la squalamine ont été également synthétisés et décrits dans la littérature. Récemment dans notre laboratoire, des analogues de la squalamine ont été synthétisés par une méthode d'amination réductrice au titane permettant de greffer des chaines polyaminées sur un stérol désiré en une seule étape [14-15]. Parmi ces composés, on peut citer le cas du dérivé DAS-1, qui présente des activités comparables à la squalamine sur différentes souches cliniques, avec des CMIs variant de 1 à 4 µg/mL [10]. L'étude relation structure activité des nombreux dérivés synthétisés a démontré que le groupement sulfate n'était pas indispensable pour les activités antimicrobiennes alors que la chaine polyaminée jouait un rôle capital dans ces activités. Il a été également rapporté que les dérivés 3β-aminostérols étaient plus actifs que les dérivés 3α-aminostérols [16].

L'objectif de ce travail est d'améliorer les activités antimicrobiennes des analogues synthétisés de la squalamine et de valoriser l'utilisation de ces dérivés pour des applications en médecine humaine. Dans ce travail, nous avons synthétisé de nouveaux dérivés 3,20-bis(polyaminostéroïdiens) présentant un nombre de charges positives important, en utilisant la progestérone comme substrat de départ, avec des rendements chimiques variant de 18 à 82 % et d'excellentes diastéréoselectivités. Nous avons pu évaluer les activités

antimicrobiennes et déterminer les CMIs sur un large panel de souches bactériennes de référence et cliniques variant de 2.5 à 40 µg/mL. Nous avons ainsi pu remarquer une corrélation entre la longueur de la chaine polyaminée et les activités antibactériennes observées avec une activité maximale pour les composés ayant une longueur de chaine carbonée comprise entre 6 et 8 carbones sur *E. coli* (article 2).

Dans un travail connexe, nous avons pu déterminer les CMIs de la squalamine, du dérivé DAS-1 et d'antifongiques classiques sur un panel de souches cliniques de levures (Candida et Cryptococcus). Des CMIs homogènes ont été obtenu pour la squalamine et le dérivé DAS-1, comprises entre 8 et 16 µg/mL et de 1 et 2 µg/mL, respectivement (Article 3). Nous avons également pu montrer que la squalamine et le dérivé DAS-1 induisaient un efflux d'ATP progressif suggérant que la membrane constitue la cible de ces molécules. Cependant, les CMIs obtenues restant relativement élevées, nous avons alors envisagé d'utiliser ces derniers pour des traitements sous formes de topiques locaux ou en tant qu'agent désinfectant.

La colonisation nasale et cutanée à *S. aureus* est responsable de nombreuses infections. La décolonisation est ainsi préconisée lors d'opérations chirurgicales lourdes afin de réduire le risque de développement d'infections post opératoires. La décolonisation est réalisée au moyen d'antibiotiques et/ou de solutions d'antiseptiques. Néanmoins, l'utilisation d'antibiotiques a conduit à l'apparition de résistance. Dans le cas de la squalamine, il a été démontré que le mécanisme d'action de cette molécule est de type "mécanique" sur les

bactéries Gram positive ce qui réduit la probabilité de développer des résistances à la squalamine. Dans cette optique, la squalamine et les dérivés 3.20-bis(polyaminostéroïdiens) (DAS-2) ont été formulés sous forme de pommade pour une application locale. Nous avons développé un modèle de décolonisation cutanée chez les souris et des pommades contenant 1% de principe actif (squalamine et DAS-2) se sont montrées efficace dans la réduction de la colonisation cutanée à *S. aureus.* L'application unique de la pommade de squalamine et de mupirocine a permis de réduire de 4 Log et 1.3 Log la colonisation cutanée à *S. aureus* en 1 h, respectivement (article 4).

Par ailleurs, les dérivés aminostéroïdiens se sont montrés très actifs sur les souches cliniques multirésistantes isolées à partir des malades mucoviscidosiques. Les patients atteints par cette maladie ont recours systématiquement à des inhalations répétées d'antibiotiques et d'agents mucolytiques, au moyen de nébuliseurs. Les souches du patient se retrouvent ainsi dans le matériel de nébulisation et deviennent ainsi une source potentielle d'infection chez le patient par la flore microbienne contenue dans leur propre tube respiratoire. De nombreuses souches bactériennes ont été isolées chez les malades atteints par la mucoviscidose et plus particulièrement *P. aeruginosa, S. aureus.* Dans le but de prévenir la réinfection du patient par son propre matériel, la désinfection des nébuliseurs est conseillée, diverses recommandations existent, certaines préconisant l'utilisation de solution d'hypochlorite de sodium ou de solutions de vinaigre. Dans cette optique, une méthode de désinfection des nébuliseurs simple et

rapide a été mise au point *in vitro* au laboratoire. Les nébuliseurs sont infectés par des souches de bactéries (*S. aureus*, *P. aeruginosa*) et de champignons de référence (*C. albicans*) puis désinfectés par immersion dans des solutions contenant de la squalamine. La squalamine à 0.5 g/L a pu réduire de 5 Log les bactéries et de 4 Log les champignons en 20 min. Concernant *A. niger,* la dose fongicide est de 2 g/L durant 6 h. Fort de ces succès, des cachets solubles de squalamine ont pu être formulés et utilisés au laboratoire afin de simplifier la méthode de désinfection (article 5).

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Chapitre I

Article 1: Revue

Polyamines metabolism, toxicity and potent therapeutical use

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Résumé

Dans cette revue, nous avons répertorié les rôles biologiques des polyamines ainsi que les activités biologiques relatives telles que leurs propriétés antivirales, antimicrobiennes et leur toxicité afin d'envisager leur possible utilisation en thérapeutique humaine. Les polyamines sont ainsi des molécules polycationiques possédant des chaînes carbonées, espacées par des groupements aminés. À ce jour, un très grand nombre de dérivés polyaminés ont été isolés et caractérisés. On peut citer les polyamines aliphatiques telles que la putrescine, la spermidine, la spermine et l'agmatine. D'autres polyamines ont été découvertes chez le requin *squalus acanthias* plus complexe telles que des polyaminostérols comme la squalamine.

Les polyamines possèdent diverses activités biologiques, la squalamine présente ainsi des activités antiangiogéniques, des études ayant démontré que cet aminosterol est bien toléré à une dose de 500 mg/m^2/jour pendant 5 jours alors qu'une dose plus élevée (700 mg/m^2/jour) produisait une hépato toxicité. Des activités antivirales ont été récemment rapportées pour la squalamine, la spermine oxydée (0.82 mM) et les céragenines (10 µM), la spermine oxydé étant capable d'inactiver le virus de la vaccine par la formation d'acroleine. De nombreuses polyamines présentent des activités antiparasitaires, on cite le cas des dérivés de l'artemisin-spermidine qui sont actifs sur *P. falciparum* chloroquine sensible 3D7 avec une IC_{50} comprise entre 0.21 to 0.76 nM. Les activités antifongiques des polyamines sont nombreuses, les cholesterolyl hydrazones tels que la

cholesteryl tosylhydrazone active sur *Candida albicans.* Enfin, les activités antibactériennes de nombreux analogues de la squalamine et des céragenines ont été largement étudiées. La squalamine et ces analogues sont très actifs sur les bactéries multirésistantes. Les charges positives portées par la chaine carbonée des aminostérols interagissent avec les groupements phosphates des lipopolysaccharides de la membrane des bactéries Gram négative. En revanche, le mécanisme d'action est de type dépolarisation membranaire sur les bactéries Gram positive suivi d'une libération du contenu intracellulaire. D'autres applications ont été envisagées tel que la transfection de cellules et la décontamination du matériel médical. Les céragenines sont aussi efficaces *in vitro* contre les biofilms de bactéries Gram positive et Gram négative sur les cathéters. La squalamine et les dérivés aminostéroïdiens ont été utilisés en application locale sous forme de pommades, capables de réduire significativement et rapidement la colonisation cutanée à *S. aureus* sur un modèle animal. D'autres applications peuvent être envisagé telles que la désinfection du matériel médical et le traitement des infections pulmonaires par voie aérosol.

ARTICLE 1

Polyamines metabolism, toxicity and potent therapeutical use

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Abstract

In recent years, extensive researches have emphasized the fact that polyamines conjugates acting as possessing antitumor, antimicrobial candidates are becoming important in the polyamines field. In this review, a general design strategy of polyamine conjugates as well as recent progress in both fundamental mechanism studies and potent therapeutic use as anti infectious agents are provided for the readers.

Keywords: Polyamines, Antimicrobial activity, Polyaminosterol

I- Introduction

Polyamines are polycationic molecules at physiological pH possessing a hydrocarbon backbone and multiple amino groups [1]. In 1678 Leeuwenhoek discovered the first polyamino substance in human semen which was named spermine [2]. Thus, Spermine is a polyamine involved in cellular metabolism found in all eukaryotic cells. It is also found in a wide variety of organisms and tissues and is an essential growth factor in some bacteria. Moreovere, spermine is associated with nucleic acids and is thought to stabilize helical structure, in particular, in viruses. Since spermine's discovery, four aliphatic polyamines have been biologically discovered i.e. putrescine, spermidine, spermine and agmatine [3]. These polyamines have been indifferently isolated from bacteria issued both from microbial flora of gastrointestinal tract and also from eukaryotic cells of animals or humans. Thus, cadaverine and putescine are both produced by the breakdown of amino acids such as lysine. However, these diamines are not purely associated with putrefaction, they are also produced in small quantities by living beings and are partially responsible for the distinctive odors of urine and semen [4]. On the other hand, spermidine is a polyamine involved in cellular metabolism. Its known actions include: inhibition of neuronal nitric oxide synthase, nNOS [5], Assisting the in vitro process of transcribing RNA via stimulation of T4 polynucleotide kinase and T7 RNA polymerase activity [6], Regulation and promotion of plant growth - as a polyamine plant growth regulator it is also a plant hormone-promoting somatic

embryogenesis [6-13] Spermidine has also been found to reduce the amount of aging in yeast, flies, worms, and human immune cells by inducing autophagy [14] Finally, spermidine is also present in high amounts in broccoli and cauliflower, putrescine in the citrus fruits, whilst these polyamines are present in meat [15]. Polyamines concentrations are variable in the different considered cellular tissues but high concentrations were generally encountered in tissues having high turnover. Polyamines have been demonstrated to be essential for cell growth and protein synthesis [16] and their content is regulated by biosynthesis, degradation and transport [17].

Emergence of multidrug resistant pathogens was responsible of numerous microbial infections and inefficacy of numerous antimicrobial therapies [18] have induced a need for the research of new classes of polyamino antibiotics [19]. In this review, we will focus our interest towards polyamines biological properties and the recent development of new polyamino antimicrobial agents.

Polyamines synthesis in cells

Polyamines biosynthesis is realized by three enzymes namely ornithine decarboxylase, putrescine amino-propyl transferase and spermidine aminopropyl transferase (Figure 1). Thus, putrescine is synthesized by ornithine decarboxylation, whereas the others polyamines are produced from putrescine by propylamine group fixation under the action of spermine and spermidine synthase. In this later case, the donors of propylamine groups are S-adenosyl S-methyl

homocysteamine compounds derived from S-adenosyl methionine [16]. Concerning agmatine, this later is produced by decarboxylation of arginine. Finally, polyamines may be metabolized by many oxidase and acetyl transferase enzymes allowing either their interconversion or elimination [16].

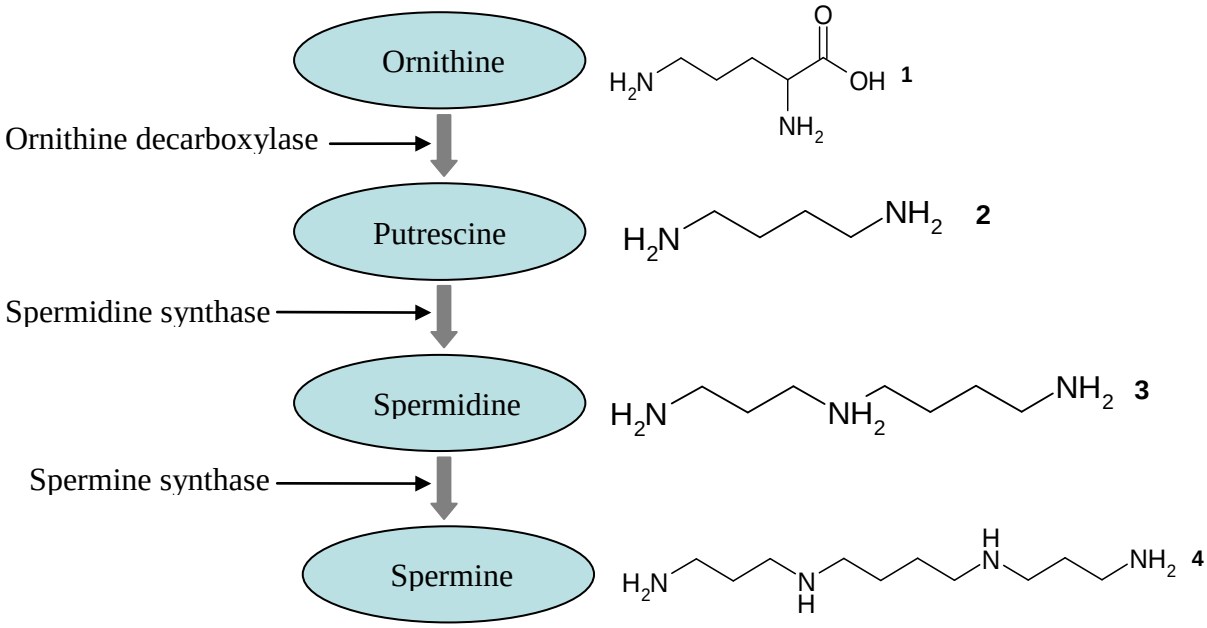

Fig. (1): polyamines synthesis

Polyamines roles and properties

Polyamines are ubiquitous compounds with different properties (figure 2) such as growth cell, cellular reparation, gene transcription, protein and nucleic acid synthesis [16]. Polyamines have been widely studied for their implication in cancer [20]. Indeed, among all the envisioned approach for preventing cancer development, a reduction of intracellular polyamines synthesis has been suggested, but this hypothesis remains limited since cancer cells corrected automatically their increasing uptake of exogeneous polyamines. Polyamines possess positive charges and can serve as electrostatic bridges between negative phosphate charges and others polyanions such as DNA and RNA [1;16]. Thus, polyamines are well known to stabilize DNA-chromatine complex and it has been observed that chromatine and DNA modification occurs in polyamine-depleted cells. However polyamines accumulation led to apoptosis through an oxidative stress process overproducing hydrogen peroxide during polyamine catabolism. Nevertheless, addition of difluoromethyl ornithine, an ODC inhibitor, lowered apoptosis rate in depleted cells. On the other hand, polyamines play a role in cell proliferation by interacting with nuclea phosphoprotein P53 involved in different gene regulation during growth and cell death. In this context, it has been demonstrated that polyamines depletion by difluoromethyl ornithine DMFO led to an increase of P 53 gene expression *in vivo* and *in vitro* [16]. Polyamines play a crucial role in growth and multiplication of prokaryotic cells [1]. Thus, polyamines promote for instance growth

of many bacteria such as *Haemophilus influenzae, Neisseria perflava* whereas they lead to an inhibition of Gram positive bacterial growth at high concentration. Polyamines are also important in bacteria outer membrane functions particularly in porins, such as in *E. coli*, where putrescine and spermidine bind to aspartic acid of porins OmpC and OmpF producing a charge modification of the pore decreasing the outer membrane permeability [1]. Furthermore, polyamines can interact with phospholipids in the membrane. In this context, spermine and spermidine are able to regulate ionic canal membrane activity blocking particularly potassium channels or glutamate receptors. Polyamines were also known to stabilize membrane cells by modulating epidermal growth factor EGF [16]. In the case of an Helicobacter infection, bacteria decrease macrophage survival via modulation of polyamines biosynthesis in host cells [1]. Thus, transcription of a proto oncogene, c-myc, upregulated in macrophages which bind with ODC promoter induces an increase of ODC expression and macrophage apoptosis. Furthermore, in a mouse model, Helicobacter causes apoptosis by membrane depolarization and caspase activation through a production of H_2O_2 by polyamine oxidase expression (PAOh1) [1]. On the other hand, it has been demonstrated that polyamines may also stimulate gene transcription and translation due to their interactions between nucleic acids and polycations in the presence of Mg^{2+} at physiological concentration.

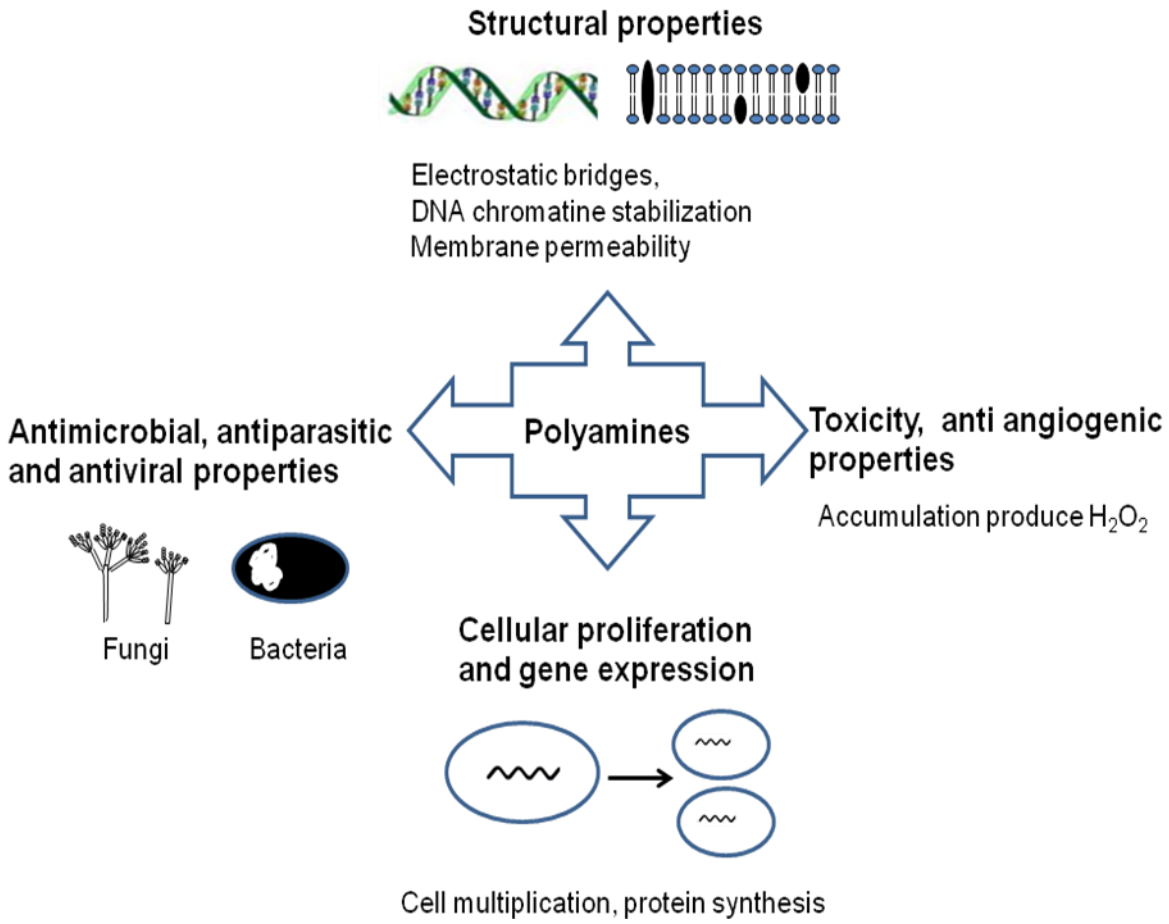

Fig. (2). polyamines roles and properties

Polyamine regulation

The level of intracellular polyamines in bacteria is regulated during the synthesis, catabolism and elimination process. Thus, three enzymes regulate polyamines metabolism namely ornithine decarboxylase (ODC), acetyl CoA spermidine/spermine N acetyl transferase (SSAT) and S-adenosyl S-methyl decarboxylase (SAMdc). Concerning ODC, its activity is controlled at gene transcription, translation and mRNA degradation and finally at its catabolism. ODC activity was controlled by regulating proteins named ornithine

decarboxylase antizyme (OAZ) generally induced after intracellular accumulation of polyamines and which forms a complex with ODC inducing an inhibition of this later and its catabolism. An increase of the intracellular polyamine level induces activation of acetyl CoA spermidine/spermine, N acetyl transferase (SSAT) and consequently acetylation of polyamines and putrescine degradation by diamine oxydase preventing polyamines accumulation. It is reported that polyamines negatively regulated S adenosyl S methyl decarboxylase SAMdc at the mRNA transcription and translation [16]. For example, in *E. coli*, ornithine decarboxylase is reversibly inhibited by either putrescine or spermidine. ODC may also be regulated at the transcription level by cyclic AMP and cAMP receptor protein complexes [1].

Polyamines transport in prokaryotic cells

Polyamines transporters were characterized firstly in *E. coli* and were encountered in many pathogens such as Gram positive, Gram negative bacteria, archae, yeasts and protozoa [1]. For example, in *E. coli*, polyamines transport includes two ABC transporters which are selective for either putrescine or spermidine and two antiporters (PotE and CadB), the first exchanging putrescine for ornithine and the second exchanging lysine and cadaverine (Figure 3). Polyamines ABC transporter genes are organized as four gene operons and designed as potABCD (spermidine uptake) and potFGHI (putrescine uptake) [1;17].

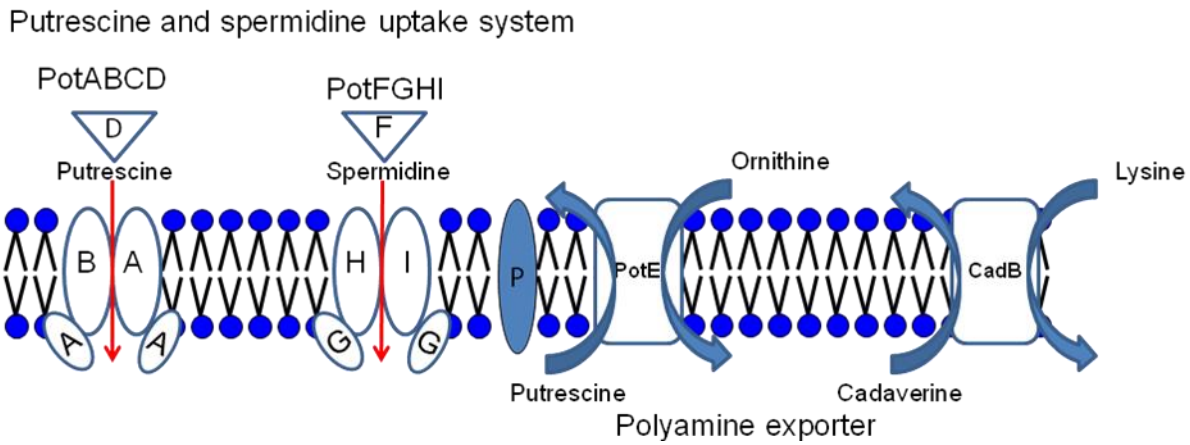

Fig. (3): polyamine transport systems in *E. coli*

II- Toxicity

Despite polyamine plays a crucial role in cellular growth, addition of polyamines (spermine, spermidine) to culture medium in presence of foetal calf serum inhibited human cell growth. Thus, it is reported that polyamine toxicity at high concentration may be the result of formation of high amounts of hydrogen peroxide during interconversion steps and consequently due to the oxidative stress (Figure 4) [16]. Thus, the concentration necessary for cell inhibition was 30 µM for spermine, 15 µM for acrolein 0.4 mM for H_2O_2. It is reported that aldehyde dehydrogenase can prevent this side-effect of spermine as well as in the case of spermidine [21]. A similar toxicity effect was encountered using spermine whatever the type of treated cell (L11210 and NIH3T3 cells) suggesting in this later case a potent common spermine's toxicity mechanism [21]. On the other hand, polyamine cytotoxicity appears highly correlated with the concentration of the formed acrolein [21] as reported in numerous studies such as, in the plasma of chronic renal failure patient where the level of spermine and spermidine decreased whereas the acrolein concentration increased [22].

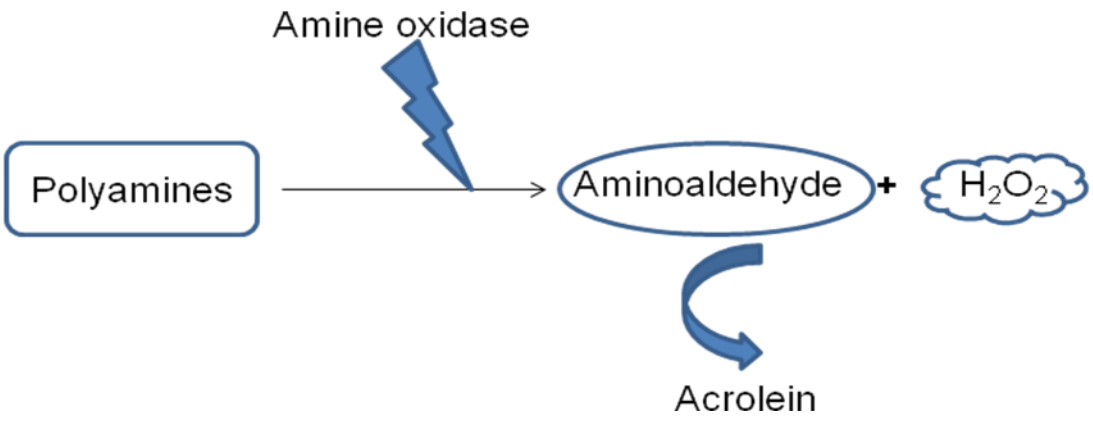

Fig. (4). Polyamines oxidation

Cytotoxicity of polyamidoamine

Polyamidoamines (PAAs) which are polymers synthesized by the polyaddition of primary monoamines or secondary amines can bind heparin and be used as drugs carriers. In this context, cytotoxicity of six PAAs (Figure 5) has been studied for their use as DNA delivery system. Most of PAAs were not cytotoxic against HepG2 cells and HL60 cells but a co-monomer methylene bisacrylamide, dimethylene diamine one PAA (NG30) exhibited significant cytotoxicity (IC 50 = 400 µg/mL) similar to 22 kDa polylysine (IC50 = 300 µg/mL). In this case, polycations disrupted cell membrane and the cytotoxicity has been associated to their ability to form a sTable complex with DNA [23].

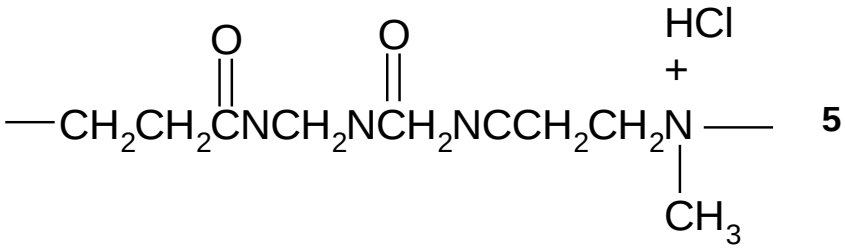

Fig. (5): Example of polyamidoamine structure

On the other hand, polyamines are important in the regulation of cells proliferation during neurogenesis. Thus, *in vitro* mature cultures exposed to 1-500 µM spermine, produce doses-dependent death of granule cells, with half maximal effect observed at a concentration below 50 µM. In this area, putrescine appears to be less toxic than spermine (500 µM) whereas spermidine toxicity was half than observed for spermine. Spermine toxicity can be prevented by competitive CGP 39551 and noncompetitive MK-801 which are N-methyl-D-aspartate (NMDA) receptor antagonist [24]. A partial prevention against spermine toxicity may be also obtained using simultaneous free radical scavengers or through inhibition of the free radical generated by the nitric oxide synthase effective against glutamate toxicities. In culture conditions, spermine toxicity was absent when glutamate toxicity was reduced. Spermine exposure induces a progressive accumulation of glutamate in the medium granule cell cultures caused by glutamate leak after cellular death. Finally, polyamines are toxic on granule cells in culture and only prevented by simultaneous presence of MK-801 and CGP 39551 [24].

Cytotoxicity of bisnaphthalimido polyamines derivatives

Naphthalimides and bisnaphthalimides (Figure 6) were known to intercalate DNA and recently reached a phase I cancer clinical trials. Thus, Pavlov et *al.* have studied toxicity of bis-naphthalimidopropyl spermidine, spermine and oxa-spermine against human breast cancer MCF-7 cells encountering IC50 values of 1.38, 2.91 and 8.45 µM, respectively [24]. Measurement of DNA binding properties indicates higher affinity of bis-naphthalimidopropyl polyamines to calf thymus DNA with C50 value (drug **c**oncentration giving a 50 % decrease in the fluorescence of the bound ethidium bromide) ranging from 0.07 to 0.12 µM lower to those observed for spermine and spermidine (C50 = 0.65 µM) [25].

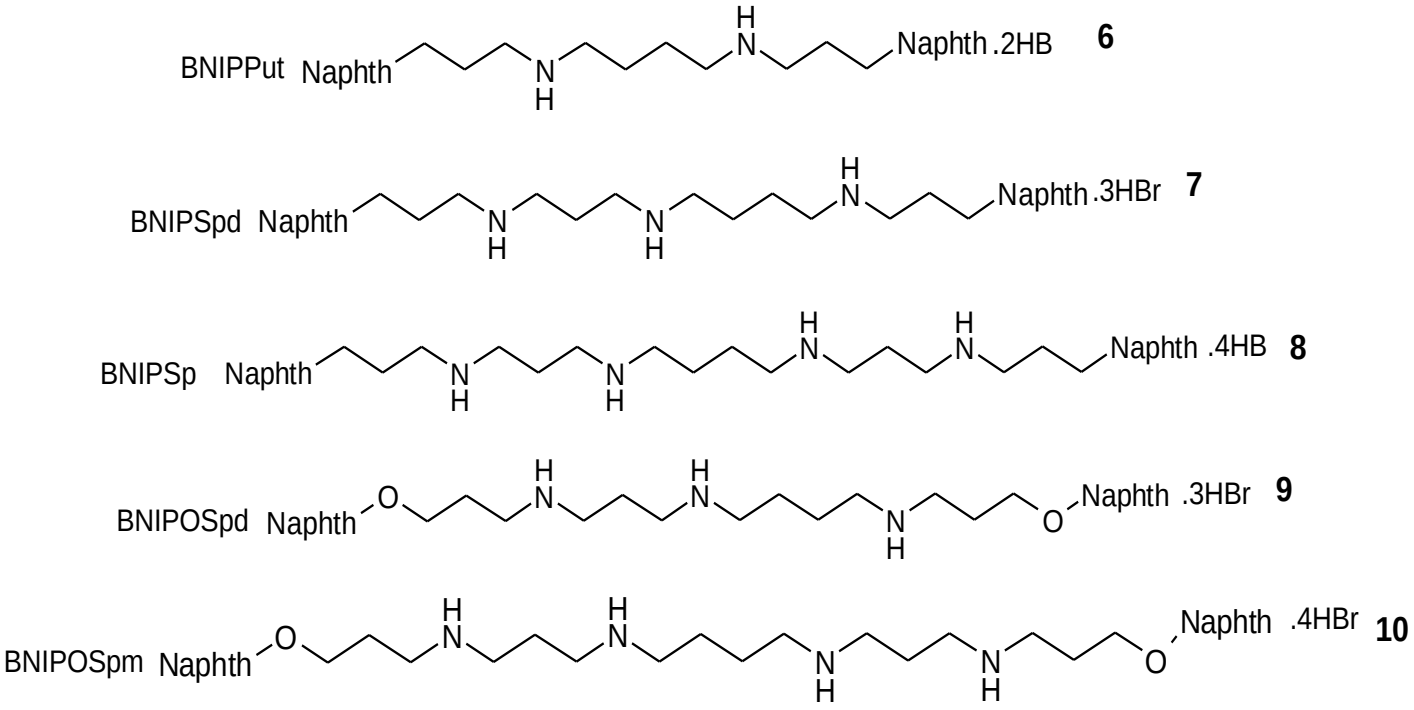

Fig. (6). Structure of bisnaphthalimido derivatives

In this context, Dance et *al.* recently reported the synthesis of new bisoxynaphthalimidopolyamino derivatives (Figure 7) demonstrating that they are less cytotoxic than bisnaphthalimido spermine and bisnaphthalimido spermidine suggesting that the nature of the polyamine chain linking to the naphthalimido rings plays a crucial role with respect to their cytotoxicities (Table 1) [26].

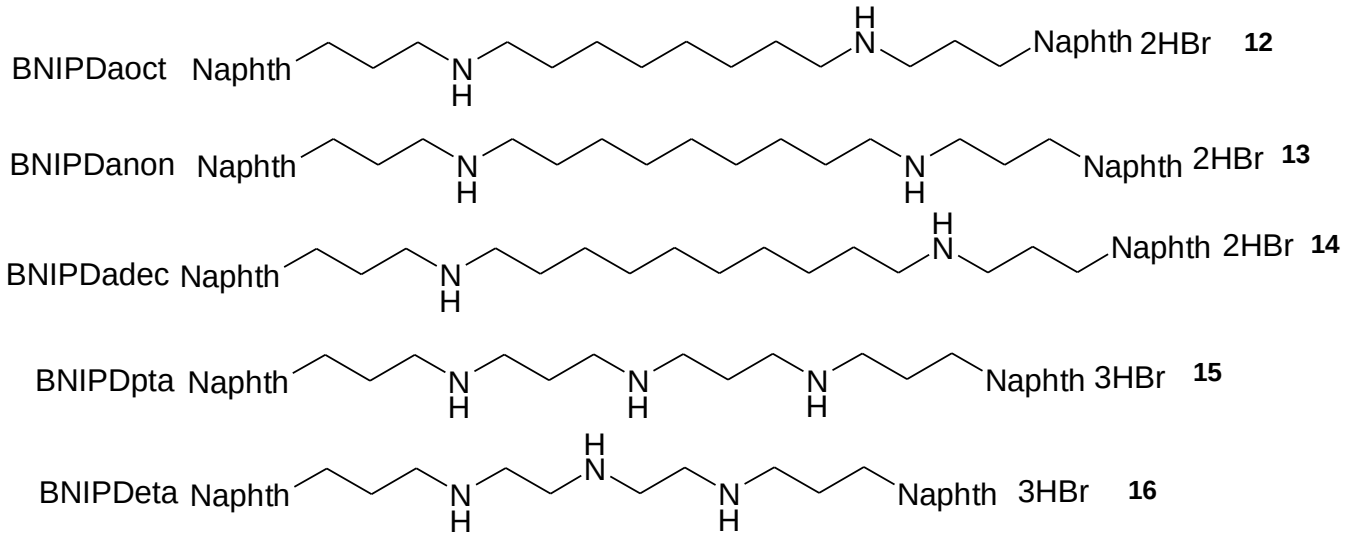

Fig. (7). Chemical structure of bisnaphthalimidopropyl derivatives

Table 1. Cytotoxic effect of bisnaphthalimido derivatives against MCF cells.

Compounds	IC 50 (μM)
Bisoxynaphthalimidopolyamine spermine	29.55
Bisoxynaphthalimidopolyamine spermidine	27.22
Bisoxynaphthalimidopolyamine putrescine	> 50
Bisnaphthalimido spermine	5.5
Bisnaphthalimido spermidine	0.73

On the other hand, Oliveira et *al.* reported the synthesis of numerous bisnaphthalimidopropyl derivatives and their evaluation for their cytotoxicity against *Leishmania infantum* and colon cancer cells CaCo-2 with IC_{50} values varying from 0.47 to 1.54 µM and 0.15 to 22.7 µM, respectively suggesting a potent antitumoral and antiparasitical use [27]. Recently, another class of polyamino derivatives belonging to the methoctramine family has been reported to be cytotoxic at micromolar concentrations, the sensibility being highly dependent on the nature of the considered cell lines and the length of the inner carbon chain. Thus, against HL 60 leukaemia cells, except for one compound, the IC 50 values were superior to 100 µM whereas in the case of SH-SY5Y neuroblastoma and H9C2 cardiomyoblasts, the cytotoxicity increased significantly with the increase of the spacing group suggesting that lipophilicity plays a crucial role for the encountered toxicity (Table 2).

Table 2. Cytotoxicity of tetramines against different cell lines.

General structure: R-CH_2 - NH- $(CH_2)_6$- NH-$(CH_2)_n$ - NH- $(CH_2)_6$ - NH- CH_2-R

R: 2-methoxybenzyl.

Entry	Spacing (n)	IC 50 (µM) against HL60	IC 50 (µM) Against SH-SY5Y	IC 50 (µM) against H9C2
1	5	> 100	> 100	45
2	6	> 100	> 100	41
3	8	> 100	87	34
4	10	> 100	76	16
5	12	55	18	3

Furthermore, when methoctramine and tetramines containing 12 carbon spacer were added to HL 60 cells, a decrease of ornithine decarboxylase activity, the first enzyme implicated in polyamine biosynthesis, was observed. Finally, it has been also demonstrated that these derivatives do not activate caspase protease which characterizes apoptosis [28]. Recently, a new family of bisnaphthalimidopropyl polyamines (BNIPPs), has been synthesized by incorporating polyamine as linkers connecting two naphthalimido ring moieties. The cytotoxicity of bisnaphthalimidopropyl spermidine (BNIPSpd) was determined against Caco-2 and HT-29 cells with IC50 values varying from 0.15 and 1.64 µM, respectively after 48 hours exposure. It has been encountered that BNIPSpd treatment (> 0.5 µM during 4 hours) induces an increase of caspase 3 protein expression indicating chromatin condensation instability and DNA fragmentation typical of apoptosis. Furthermore, addition of a few amount of BNIPSpd (> 0.01 µM) to Caco-2 and HT-29 cells reduces significantly intracellular polyamine levels (spermine and spermidine) which have been already mentioned as essential for the cell growth [29]. Concerning, N benzyl spermidinyl linked to bis (1,3,5-thiadiazinane-2-thiones) class derivatives, their toxicity (LD 50) ranged from 1.75 to 52.54 µM and is principally related to their mechanism of action which could be due to the interference with natural polyamines metabolism [30]. Otherwise, numerous 5,6-heteroaromatically pyridine-2,4-diamines have been widely evaluated against human

cancer cells *in vitro* and most of the compounds lead to a weak cell growth inhibition with respect to ellipticine [31].

Toxicity of spermine in cultured human cerebral cortical neurons

More particularly, the toxicity of spermine and putrescine has been widely investigated against human cortical cultures containing neurons and glia, demonstrating that they do not present significant cytotoxic effects at 0.3 to 2 mM concentrations during a 8 days treatment whereas addition of foetal calf serum generates immediately toxic products due to an oxidative metabolism process leading to the cellular death. Simultaneous addition of spermine (2 mM) and glutamate (5 mM) produce, an *in vitro* neuron cell death whereas these compounds separately added do not lead to noticeable toxic effect. More precisely, morphological changes demonstrate that spermine led to only to a neurone death whereas glutamate was able to induce astrocytes nuclei swelling. Pharmacological studies performed with two NMDA receptor antagonists (ifenprodil and MK- 801) led to a spermine cytotoxic effect (2 mM) involving a N-methyl D aspartate receptor activation mechanism [32].

Numerous spermine synthetic analogues have been prepared such as N1,N12-bis (ethyl) spermine reducing the level of spermine in mouse FM3 cells, their accumulation inducing also an inhibition of the cell growth after incubation for several days. Deficient polyamine cells inhibited spermine / spermidine acetyl transferase activity and stimulated polyamine uptake. While N1,N12-bis (ethyl) spermine

enhanced spermine / spermidine acetyl transferase activity and induce also an inhibition of polyamine uptake, these activity appearing widely correlated with mitochondria swelling and ATP content decrease [33].

III- Biological activities

Polyamines have been widely studied as antiinfectious agents. In this context antiviral and antiparasitic activities have been encountered even if the antimicrobial activities remain the major studied side (Figure 8)

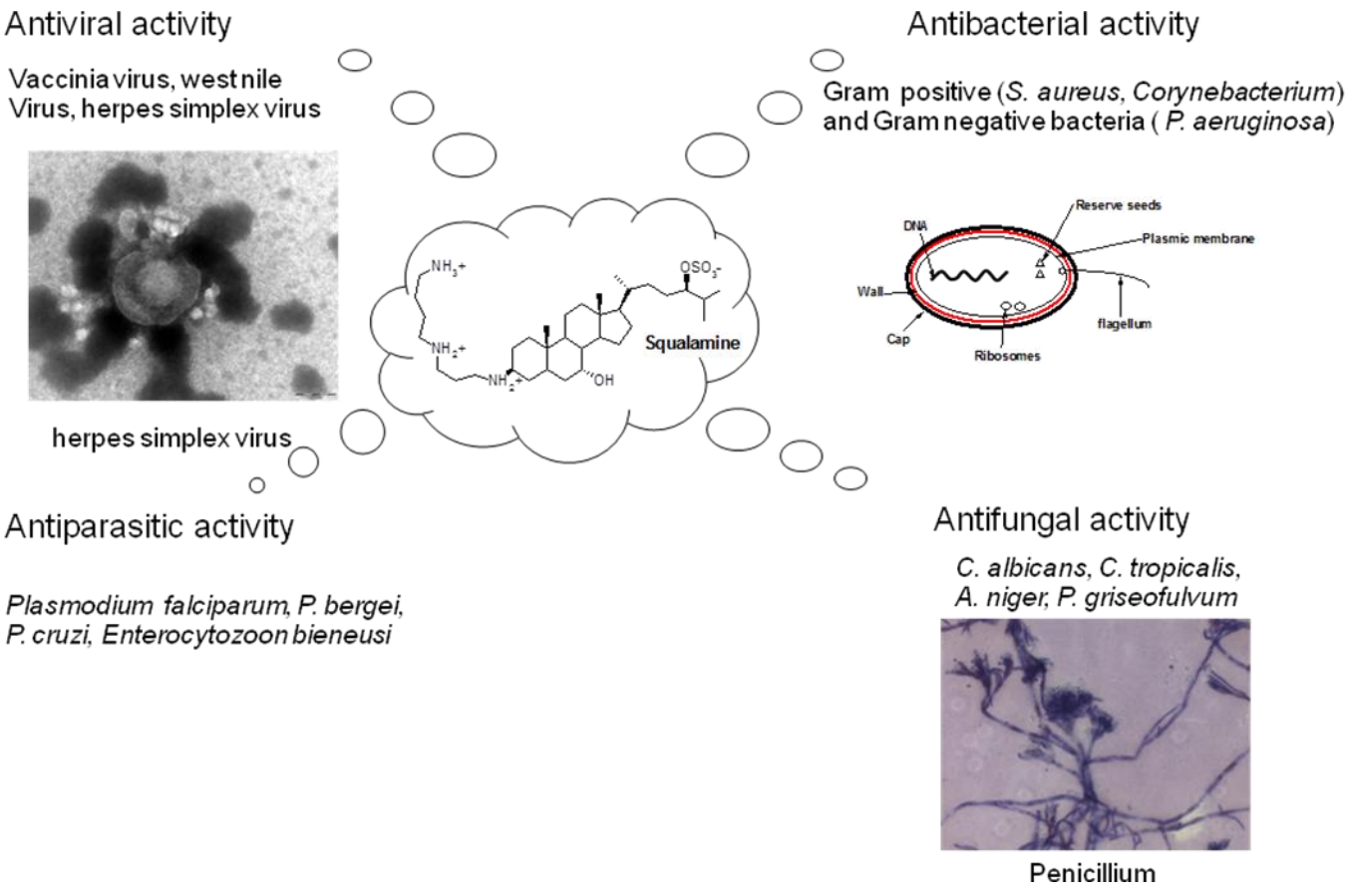

Fig. (8). polyamines activities

III-1-1 Antiviral properties

Bachrach et *al.* showed that oxidized spermine (0.82 mM) was able to inactivate vaccinia virus (10^8 plaque forming units) after incubation during 10 hours at 37 °C probably due to acrolein formation after degradation of oxidized spermine, able to penetrate into the viral particle and form a sTable complex with DNA [34]. Polyamines are also very active against coliphages of the T-odd series (double stranded DNA phages) due to their permeability towards oxidized spermine. Thus, this later (100 µg/mL during 3 hours) reduces significantly the number of plaque forming units of animal viruses such as West Nile virus (reduction of 4 logs). It is noteworthy that in the case of Newcastle disease viruses the rate of inactivation is time dependent, the authors concluding that oxidized polyamines are able to penetrate into viral particules and interact with their nucleic acid [35].

On the other hand, some N-carbamoyl and N-acyl diamine derivatives were reported to possess antiviral activity with EC_{50} ranging from 16.0 to 27.0 µg/mL against Herpes simplex virus type 1, however this active compound demonstrates cytotoxic properties and a low selectivity index (≤ 6) [36]. In this area, particular ceragenins have been reported to possess antiviral effect against vaccinia virus by inactivating 91% of vaccinia virus at 10 µM concentrations by disturbing the envelope and internal structure of viruses [37]

Very recently, Zasloff *et al.* reported that squalamine exhibits broad-spectrum antiviral activity against human pathogens (Figure 9), which

were studied in vitro as well as in vivo. Both RNA- and DNA-enveloped viruses are shown to be susceptible. The proposed mechanism involves the capacity of squalamine, a cationic amphipathic sterol, to neutralize the negative electrostatic surface charge of intracellular membranes in a way that renders the cell less effective in supporting viral replication [38]

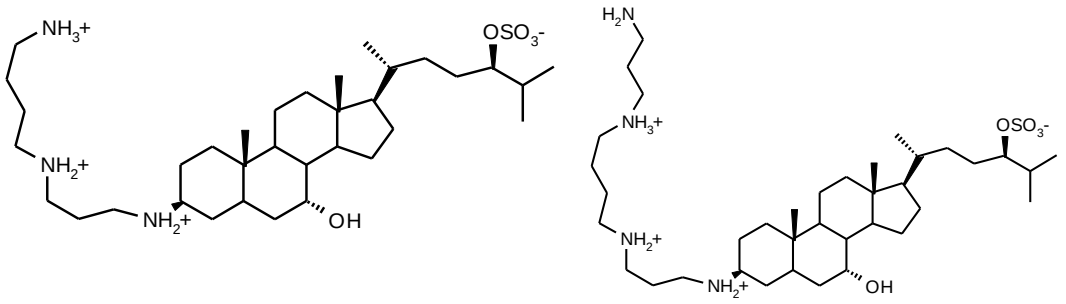

Fig. (9): Structure of squalamine, trodusquemine

III-1-2Anti parasitic activity

One of the first application of polyamine synthesis inhibition concerned parasitology. Thus, DFMO described previously is an ODC inhibitor which has been applied for the treatment of trypasonosome infections, its efficiency being due to the ODC half life time which was longer in the case of parasites (some hours) in comparison with humans (10 minutes) [16]. It has been also reported that DMFO was effective against *Plasmodium berghei* in mice [39]. Numerous polyamino derivatives have been investigated for their antiparasitic activities.Thus, antimalarial activity of artemisin-spermidine conjugates (A-B) (Figure 10) evaluated against chloroquine sensitive 3D7 *P. falciparum* strain present IC_{50} values ranging from 0.21 to

0.76 nM [39]. A series of 1, 3, 5-triazines-substituted polyamines has been also synthesized exhibiting interesting antimalarial activities against *P. falciparum* with better activities against chloroquine resistant strain K1 in comparison with chloroquine sensible line. In the case of simple substituted derivatives, it was noted that an increasing length of the central chain improves antiplasmodial activity whereas compounds possessing a cyclic central chain are less active [40]. Otherwise, Salmi et *al*. have prepared a series of 3 aminosterol derivatives and reported that they possess antimalarial properties against susceptible (3D7) and resistant (W2) chloroquine strains. IC_{50} activities ranging from 2 to 22 µM have been encountered suggesting the importance of the nature of the amino group involved [41].

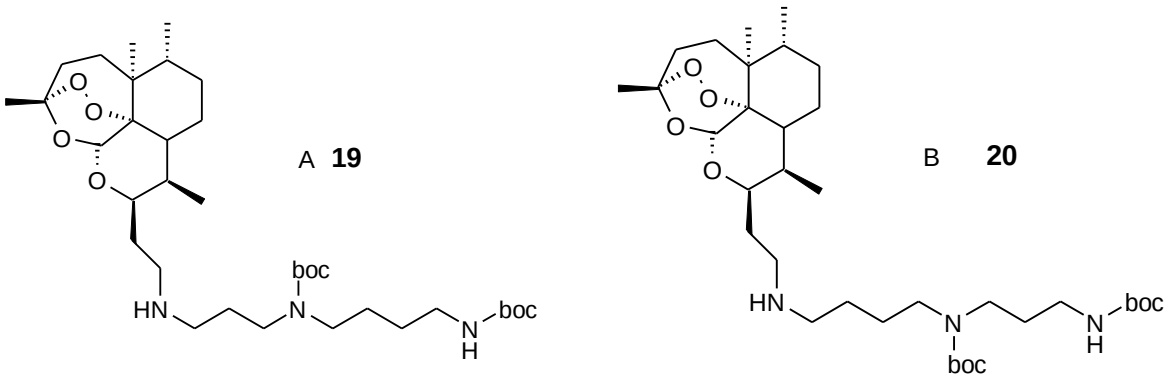

Fig. (10). Structure of artemisin-spermidine compounds

On the other hand, inhibitory properties of polyamine analogues (PG 11157, PG 11158 and PG 11302) were evaluated against an intracellular parasite *Enterocytozoon bieneusi* infection on mice. Only orally administration of PG 11157 and PG 11302 show significant inhibitory activity (96.2 to 99.6 %) better than classical fumagillin treatment (93.7%). Nevertheless these derivatives and fumagillin were

unable to totally eradicate *E. bieneusi* because spore reappearance was observed 7 days after the end of treatment. It is noteworthy that a drug toxicity was observed during the treatment but disappeared at the end of the therapy [42]. On the other hand, Coro et *al.* have investigated antimalarial activity of N benzyl spermidinyl linked bis (1, 3, 5-thiadiazinane-2-thiones) (Figure 11) which possess against *Trypanosoma cruzi* and *Leishmania donovani* IC_{50} values ranging from 3.64 to 8.69 µM and from 5.35 to 7.26 µM, respectively. Lower activities were encountered against *Plasmodium falciparum 3D7* but this family of compounds presents high cytotoxicity levels in comparison with synthesized alkyl tether analogs possessing the same amino acid residues attached to the position N^5 of the heterocyclic ring. The mechanism of action of thiadiazinane-2-thiones was reported under physiological conditions and was caused by chemical and enzymatic degradation inducing cytotoxic compounds which are toxic for mammalian cells and their ability to interact with the thiol groups present on the active site of cysteine proteinases present on the parasite [30].

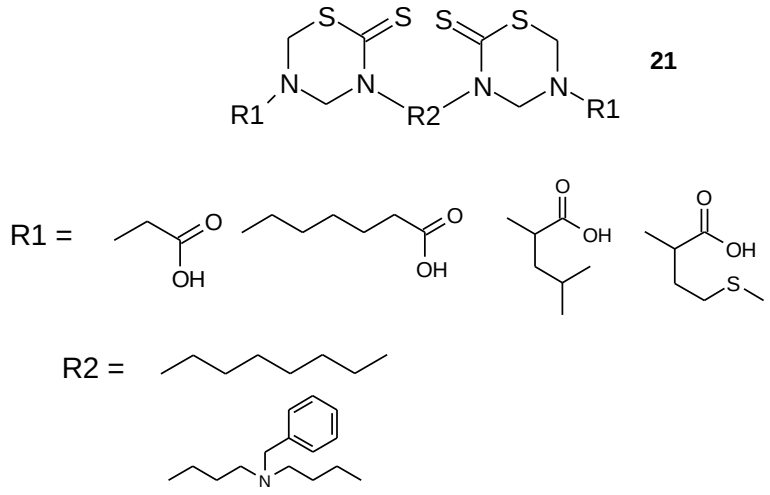

Fig. (11). Structure of N benzyl spermidinyl linked bis (1, 3, 5 thiadiazinane-2-thiones)

Recently, Lara et *al*. determined in vitro anti trypanosomatical activity of amphiphilic compounds such as ceragenins. In this context, CSA1 possesses an interesting activity against *Leishmania major* promastigotes and *Trypanosoma cruzi* trypomastigotes with LD_{50} of 4.9 and 9 µM, respectively. It has been assumed that this compound probably act by disruption of membrane integrity of the parasite [43]. Finally, antiproliferative activity of ethylene diamine derivative (Figure 12) was studied against *Leishmania* species. Thus 1,2 bis(methoxybenzyl) ethylene diamine with free amino group was very active against *L. amazonensis* and *L. major* promastigote forms with IC_{50} of 1.9 and 1.8 µg/mL, respectively. Cytotoxicity studied towards mouse peritoneal macrophages showed that this compound was not toxic at the maximum concentration tested (30 µg/mL) and it has been supposed that its anti leischmanial activity could be associated with polyamine synthesis inhibition [44].

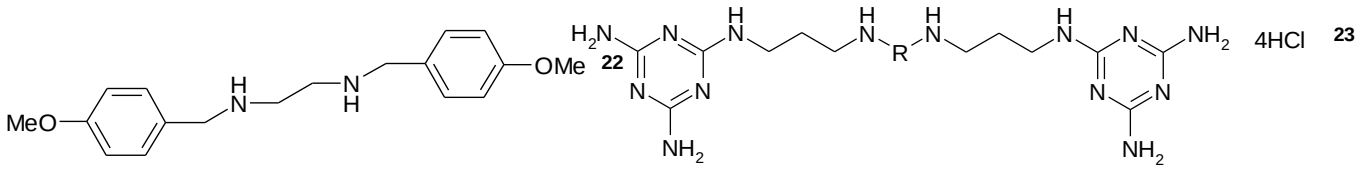

Fig. (12). Structure of 1, 3, 5-triazine-substituted polyamines (R = $(CH_2)_4$, $(CH_2)_9$, $(CH_2)_{12}$) and ethylene diamine.

III-2-3 Antifungal properties

Bis(benzyl)diamine and bis(cyclohexylmethyl)diamine were reported to be effective at 250 µM in reducing mycelial growth of *P. avenae.* Both of bis(benzyl)diamine and hydroxypyridylethylamine reduced 70% of powderly barley infection when applied as post inoculation spray at 250 µM [45]. On the other hand, aminosterol derivatives have recently attracted the interest of researchers. In 1993 Moore et *al.* reported for the first time, the antifungal properties of squalamine against *C. albicans* (MICs 4-8 µg/mL) [46]. Since this date, numerous aminosterols have been synthesized, among those compound 7-ß and 7-α aminocholestanol which are active against *C. albicans* and *C. tropicalis* even against amphotericin B resistant strains with MICs values of 1.5 and 6.2 µg/mL, respectively [47]. Various hydrazone cholesterol derivatives (Figure 13) have been also synthesized and screened for their antifungal activities such as tosylhydrazone cholesterol derivatives presenting MICs values of 1.5 µg/mL against *Candida albicans* and 12.5 µg/mL against amphotericin B resistant *C. albicans* strain [48].

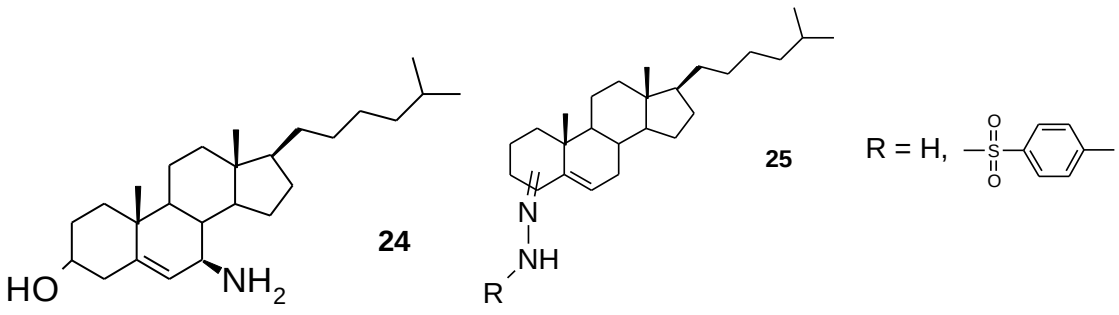

Fig. (13). Structure of tosyl hydrazone and cholesterol hydrazone

Recently, Alhanout et *al.* evaluated for the first time the antifungal activity of squalamine and aminosterol derivative ASD-1 (Figure 14) against a large panel of moulds isolated from cystic fibrosis patients. MICs ranging from 8 to 16 µg/mL for squalamine and 2-4 µg/mL for ASD-1 where encountered againts the majority of isolates resistant to classical antifungal drugs [49].

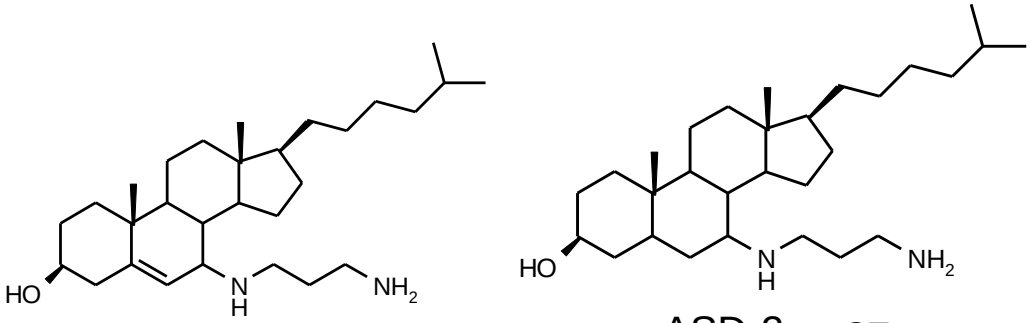

Fig. (14). Structure of ASD-1 and ASD-2

On the other hand, the efficiency of squalamine and ASD-1 has been measured against 30 yeast strains involved in blood stream infection with MICs ranging from 8-16 µg/mL and 1-2 µg/mL for squalamine and ASD-1, respectively (Table 3) [50].

Table 3. Antifugal activities of squalamine and ASD-1 (MICs mg/L)

Strains / Compounds	*C. albicans*	*C. krusei*	*C. glabrata*	*A. niger*	*A fumigatus*	*S. prolificans*	*Fusarium spp*
Squalamine	16	8	8	16	8-16	8-16	16
ASD-1	2	2	2	4	4	2-4	2-4

The mechanism of action of aminosterol derivatives on fungi remains partially unknown but time kill studies against *C. albicans* and *C. glabrata* strains indicate that the fungicidal effect was observed at 4h for ASD-1, amphotericin B and 8 h for squalamine. Measurement of ATP efflux demonstrates that this phenomenon occurs totally after 40 min for squalamine and ASD-1 whereas in the case of amphotericin B, no ATP efflux was observed indicating that aminosterol derivatives induce probably a yeast membrane disruption [50].

III-2-4 Antibacterial activity

With the extensive use of antibiotics, emergence and dissemination of resistance were observed in bacteria over the last 60 years. The search of new antimicrobial agents appeared necessary to circumvent this resistance [51]. In this context, a large variety of substances was isolated from animal species or chemically synthesized and have been evaluated for their potent antibacterial activity. Among these compounds, aminosteroid compounds such as ceragenins and squalamine derivatives present interesting antibacterial activities.

III-2-4-1 Ceragenins derivatives

Ceragenins pertain to a group of cholic acid derivatives that have been chemically modified to present common features with cationic peptides [52]. These compounds possess a wide broad spectrum of activity against Gram positive and negative bacteria [53]. Among

synthesized ceragenins, CSA1 (Figure 15) appears very efficient against multi drug resistant bacteria [54] with MICs values of 1 µg/mL against four vancomycin resistant *S. aureus* strains. It has been reported additionally that CSA1 exhibited activities against clinical isolates of *P. aeruginosa* with MICs ranging from 1 to 32 µg/mL including multidrug resistant strains. In this context, a Time kill studies analysis demonstrated a concentration dependent activity, with CSA1 at 4 * MIC achieving kill in 1 hour [55;56]. Ceragenin CSA1 exhibited also a broad spectrum activity against cariogenic and periodontopathic bacteria, which MICs values ranging from 1 to 16 µg/mL against *Streptococcus mutans* and *Porphyromonas* species [57]. Furthermore, activity of CSA1 was also measured against *Helicobacter pylori* with minimal bactericidal concentration varying from 0.257 to 8.9 µg/mL. Thus, this compound is active only at acid pH in the presence of pepsin; the authors conclude that CSA1 was resistant to degradation and inhibition by mucin and could perhaps have a potent use for the treatment of *H. pylori* infections [58]. In the same area, these compounds appeared active against *E. coli* and *S. aureus* with MICs values of 0.31 and 0.59 µg/mL, respectively. CSAs show a rapid killing bacteria, some compounds such as CSA 1 added at their MIC concentration can reduce by half bacterial population (10^5 colony forming units) from *E. coli* and *S. aureus* in 15 and 75 min, respectively [59]. Selectivity of CSAs varies depends on the structure, some of them exhibiting low activity (MICs > 5 µg/mL) with high haemolytic activities (MHCs > 100 µg/mL) while others

were very active against Gram positive and negative bacteria (MICs < 4 µg/mL) [59]. Concerning the mechanism of action of these derivatives, ceragenins possess higher selectivity against Gram negative bacteria (>10^5) than Gram positive bacteria and eukaryotic cells [54]. The bacterial membrane has been identified as the major target of these cationic, facially amphiphilic compounds including endogenous peptides [55]. Thus, it was shown that the role of the hydrophobic chain was to facilitate the self promoted transport in Gram negative bacteria [60]. On the other hand, ceragenins are able to depolarize bacterial cell membrane and produce membrane blebbing in *E. coli* [61] as well as in the case of Gram positive bacteria, *Micrococcus luteus* [54;59]. It is also reported that ceragenins with the larger number of positive charges were not necessarily the better antibacterial agents against Gram negative bacteria, for example CSA1 which contains 4 positive charges possess a better activity than other compounds containing 3 and 6 positive charges. On the other hand, since the negative charges present in the cell walls of Gram positive bacteria are different, it greatly influences antibacterial activity of ceragenins [52]. In this context, Epand et *al.* probed the interaction of ceragenins with phopholipid biplayers, by demonstrating the specific association with phopholipids, ceragenins being generally less active against bacteria with a high content of phophatidylethanolamine [54;61].

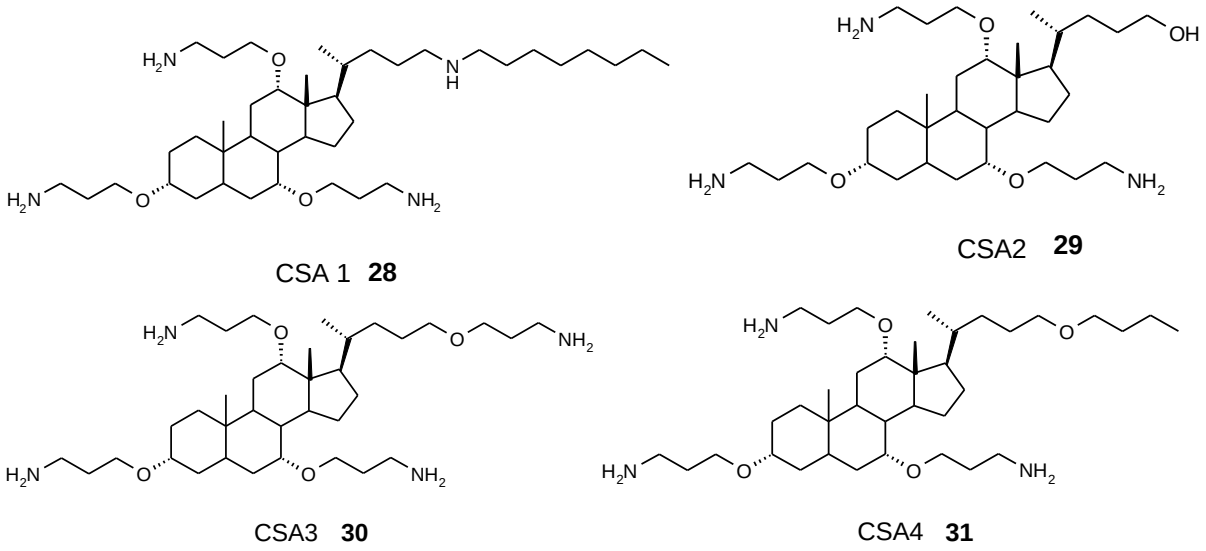

Fig. (15). Structure of cationic steroid CSA1, 2, 3 and CSA4.

In Gram negative bacteria, the permeability barrier is due to the cross-bridging between lipid A and divalent cations in the outer membrane, consequently a compound able to bind one of these constituents can disturb the organization of the outer membrane and sensitize bacteria to hydrophobic antibiotics. It was demonstrated that some cationic antibiotic peptides having low activity against Gram negative bacteria were able to permealize the outer membrane [62]. Some cholic acids derivatives permeabilized the outer membrane of Gram negative bacteria and improve activity of hydrophobic antibiotics such as erythromycin and rifampicin with FICs for compound CSA2 and CSA3 of 0.24 and 0.58 for novobiocin and 0.19 and 0.13 for rifampin (Table 4) [53]. It is reported that these compounds possess potent similar activities to polymyxin B well known to disrupt outer membrane organization via binding to the lipid A phophate moieties

of LPS via ionic interactions with the amino groups of polymyxin B. Measurement of interactions of a cationic steroid demonstrates a greater affinity towards *E. coli* (Factor 10000) than *S. aureus* due to the presence of lipid A in the outer membrane of *E. coli* [63].

Table 4. Antibacterial and haemolytic activity on Gram negative bacteria and permeabilized properties of cholic acid derivatives on *E. coli*.

Compounds	MICs against Gram negatives bacteria (µg/mL)	FICs with novobiocin	FICs with rifampin	MHCs (mg/L)
CSA1	0.8-3.7	/	/	6
CSA2	15-53.3	0.24	0.19	100
CSA3	5-25	0.58	0.13	170

Gram negative bacteria: *E. coli, P. aeruginosa, S. typhimurium, K. pneumonia.*

III-2-4-2 Squalamine and Aminosterol derivatives

Squalamine was active against a large panel of microorganisms with MICs ranging from 1 to 8 µg/mL against Gram positive and Gram negative bacteria [46].

Trodusquemine, another aminosterol isolated from the dogfish shark, structurally identical to squalamine except to the remplacement of the spermidine moiety by a spermine one, possesses better antimicrobial activity than squalamine. A series of analogs of trodusquemine was then synthesized from stigmasterol by Shu et *al.* demonstrating that analogs without 7 hydroxyl goup posses similar antimicrobial activity

and were less active than trodusquemine (Figure 8). It also appeared that 3 ß analogs exhibit better activity than 3 α analogs [64] and that the presence of a sulphate group is not essential to obtain good antibacterial activities. Many squalamine mimics were synthesised by using cholic acid, hydroxy cholic, deoxycholic acid, lithocholic acid, putrescine and spermine as starting materials; this family of compounds possess modest activity 0.5 to > 256 µg/mL, those having relatively high facial hydrophilicity exhibit strong antibacterial activity especially against Gram positive bacteria [65]. In the same area, Kim et *al.* reported the synthesis of squalamine analog from 22-hydroxy-23, 24 bisnochola-4-en- 3- one with low antibacterial activities except against *Bacillus subtilis* and *S. aureus* (3.13 µg/mL and 6.25 µg/mL, respectively) and *Micrococcus luteus* and *Streptococcus equisimilis*, (12.5 µg/mL) [66]. In the same context, Khan et *al.* synthesized a series of 7α amino 23, 24 bisnor 5α cholan 22 ol derivatives and almost compounds present a good activity against Gram positive bacteria (MICs values 1.6-25 µg/mL) whereas a weak activity (MICs value 6.3 to > 100 µg/ml) was noticed against Gram negative bacteria. These results suggest that substituents at C3 and the presence of a 7α amino group play a major role for the encountered antimicrobial activities of the compounds [67]. Khan et *al.* also reported synthesis of 3- polyamino-5ß cholane 7-α, 22- diols, concluding that antimicrobial activity is highly depending on the length and stereochemistry of the 3-amino group [68].

Kim et *al.* reported the synthesis of 3-polyamino-23,24-bisnorcholanes, this family of compound possess a modest antimicrobial activity against Gram positive and Gram negative bacteria, the most potent compound had MICs varying 1 to 16 µg/mL on *S. aureus* strains [69]. Aher and co-workers have synthesized and evaluated the biological activities of bile acid based amino sterol against numerous pathogens but with low to moderate activity against all the tested strains, except for bile acid ethylenediamine conjugate presenting similar activity to gentamicin against *S. aureus* and *Trichophyton mentagrophytes* (MICs 6.25 µg/mL) [70]. In the same context, Deoxycholic acid base spermine are active against numerous bacteria and even against vancomycin resistant *E. faecium* and methicillin resistant *S. aureus.* Thus, the authors concluded that the biological activity is highly correlated with the cationic charge and the length of polyamine chain groups [71]. Recently, new 7 aminosterol squalamine analogues were synthesized by titanium reductive amination reaction demonstrating good activities against *S. aureus*, *S. faecalis* and *E. coli* with MICs varying from 2.5 to 10 µg/mL [72]. In the same area, Salmi et *al.* synthesized a series of 3 amino and polyaminosteroid analogues of squalamine which lead to MICs varying from 6.25-100 µg/mL against various bacterial strains highly dependent on the sterol structure and the attached amino group [73]. Alhanout et *al.* demonstrated also that squalamine and aminosterol derivatives possess good activity against bacterial clinical isolates including multi drug resistant strains recovered from cystic fibrosis

patient (137 strains), MICs being included between 2-32 µg/mL and 0.5-32 µg/mL for squalamine and ASD-1, respectively, against Gram positive bacteria. Similar activities against Gram negative bacteria were encountered for these two compounds whereas higher MICs were obtained against mucoid Gram negative isolates (16-64 mg/mL) with respect to non mucoid ones (2-16 mg/mL) (Table 5) [74].

Table 5. Antibacterial activities of some aminosterol derivatives (MICs mg/L).

Compounds	Gram positive bacteria			Gram negative bacteria							
	S. aureus	***S. pneumoniae***	***E. faecalis***	***E. coli***	***P. aeruginosa***	***P. aeruginosa****	***K. pneumoniae***	***E. aerogenes***	***I. limosus***	***B. cepacia***	***A. xylosidans***
Sq	2-8	32	-	8	2-8	4	8	32	16-64	16-64	64
ASD-1	0.5-4	32	-	16	2-8	4	8-16	32	16-64	16-64	64
ASD-2	2-8	32	-	4	2-8	8	16	64	128	128	128
CSA2	4	4	3	2-37	-	-	1	-	-	-	-
CSA3	9-15	9-15	25	53.5	-	-	41.3	-	-	-	-

*P. aeruginosa**: mucoid strain

Mechanism of action of squalamine

Since squalamine is very active against a large panel of bacteria, the mechanism of action of squalamine has been investigated. Ionophoric properties of this compound have been envisioned in zwitterionic and anionic phospholipid vesicles but no significant difference in proton translocation across membrane have been observed leading to the conclusion that squalamine does not possess ionophoric activity in vesicles comprised of phosphatidylglycerol, phophatidyl choline or a mixture of these two lipids [75]. Alhanout et *al.* also were the first to report the mechanism of action of squalamine against Gram negative and Gram positive bacteria (Figure 15 and 16) [76]. On a macroscopical point of view, electronical microscopy analysis revealed formation of bleb-like projections in *P. aeruginosa* treated with squalamine whereas radiations originating from the cytoplasm were observed after treatment with colistin whereas it has been suggested that squalamine could act as this later one. On the other hand, in the case of Gram positive bacteria, treatment of *S. aureus* with squalamine caused a dramatic disruption of membrane with drained cytoplasmic content, while no morphologic changes were observed with colistin [76]. Thus, membrane integrity was measured by intracellular ATP release, an indicator of membrane lesions. On Gram negative bacteria a time dependent release was observed with a maximal efflux obtained after 20 min whereas on Gram positive bacteria, squalamine produces a rapid maximal ATP release just after 3 min. Moreover, a strong depolarization effect was observed in the

case of Gram positive bacteria whereas no effect was encountered with Gram negative bacteria suggesting two distinct mechanisms of action for squalamine (Figure 16,17) [76].

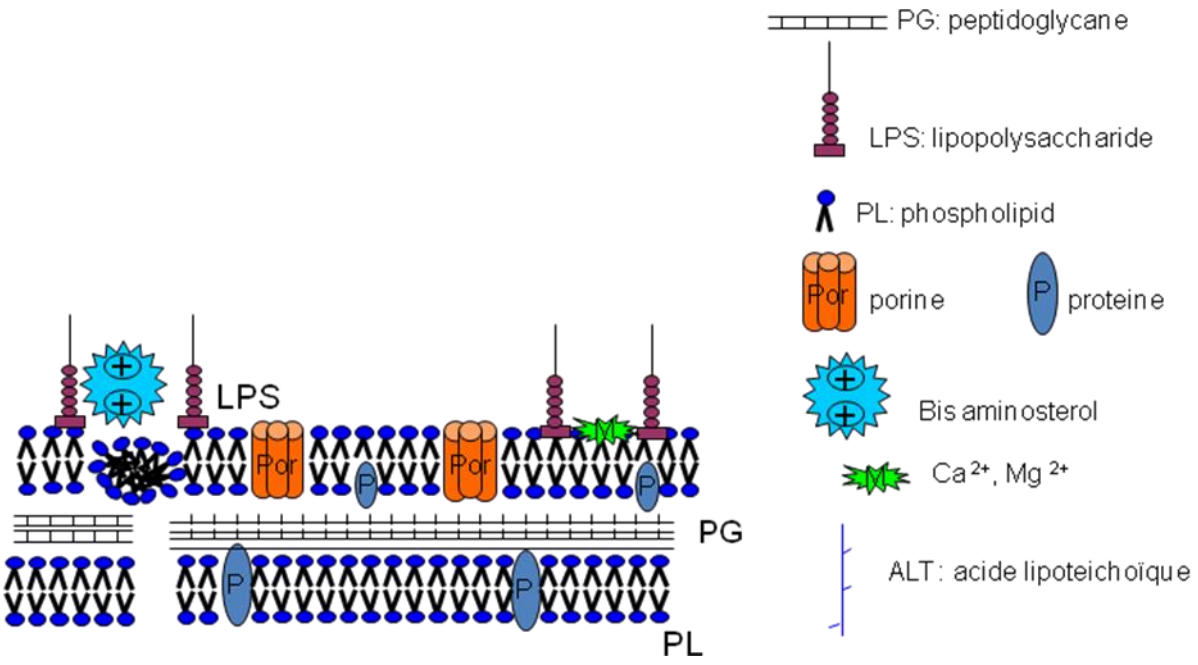

Fig. (16). Mechanism of action of bis aminsoterol against Gram negative bacteria

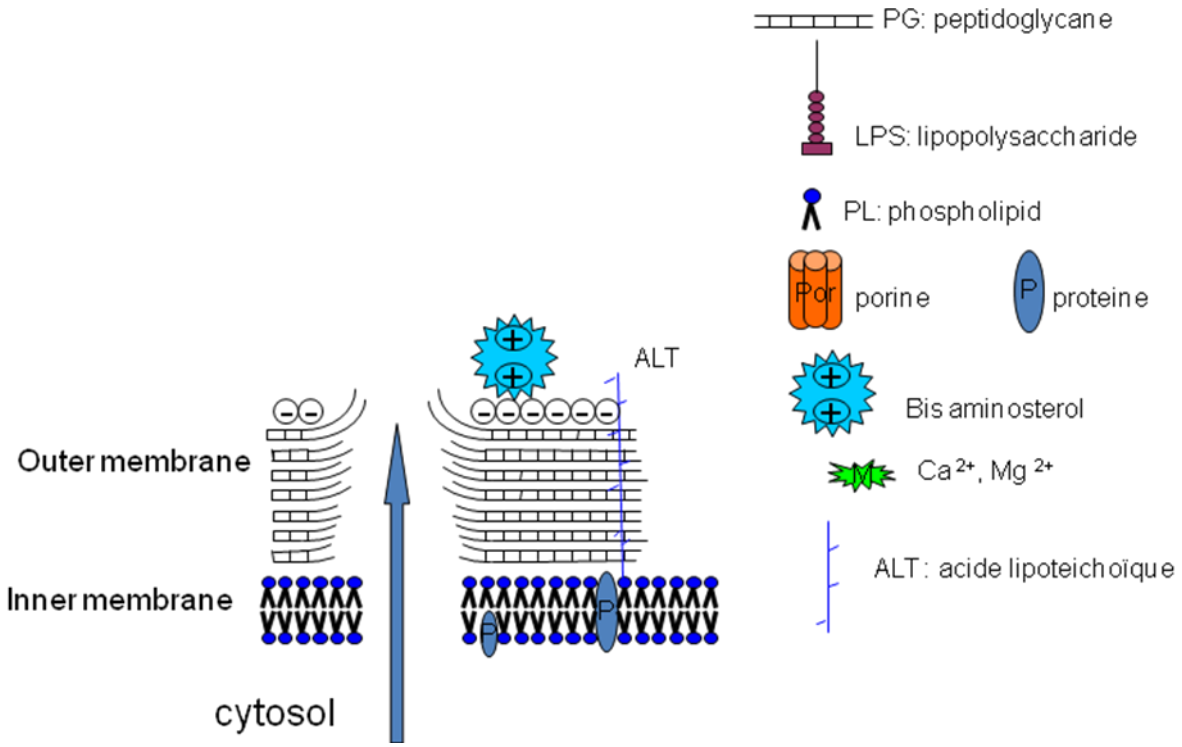

Fig. (17). Mechanism of action of bis aminsoterol against Gram positive bacteria

It is noteworthy that squalamine or colistin interaction with the outer membrane of Gram negative bacteria led to the formation of lesions with diameters of 33.3 and 9.1 nm, respectively [77]. Gram negative bacteria contain an outer membrane, formed by cross bridging between lipopolysaccharides and divalent cations (Ca^{2+} and Mg^{2+}), which constitutes an effective barrier for hydrophobic antibiotics. Many derivatives such as EDTA, some cationic peptide and polyamines can disturb membrane organization by perturbing the binding site stability [78]. Salmi et *al.* study demonstrated that addition of squalamine at 20 µg/mL lead to a release of intracellular ATP (80%). Additionally, the effect of monovalent and divalent cations (1 mM) on the antibacterial activity of squalamine was determined against *E. coli*, monovalent ions had no effect on squalamine activity outcome whereas divalent cation salts such as $CaCl_2$ and $MgCl_2$ inhibited squalamine activity. Affinity of squalamine to different bacterial and eukaryotic lipids was measured demonstrating that squalamine penetrates at low concentration to the LPS monolayer whereas a higher concentration was necessary for insertion in other monolayer containing neutral glycosphingolipids or gangliosides extracted from lymphocytes [51]. All these assumptions led to the conclusion that squalamine was able to disturb the membrane integrity of Gram negative bacteria by interaction with negatives charges of phosphate groups of the outer membrane (Figure 16) [51].

Outer membrane permeabilizing properties of squalamine

Since squalamine is able to disturb bacterial membrane integrity by increasing its permeability, an approach using this compound as a chemosensitizer agent has been improved against resistant strains. Squalamine was used at 1/5 and 1/10 of its MIC value and was able to enhance significantly the activity of chloramphenicol, tetracycline, ciprofloxacin against isogenic *E. coli* AG100 and AG100Atet strains which overproduce many drug efflux pumps and down regulate porin expression. In the *Klebsiella pneumoniae* KP55 clinical isolate, MICs was reduced by a factor 16 in presence of chloramphenicol, tetracycline, ciprofloxacin and cefepime. This result may be exploited as therapy for the development of new drug combinations against mutltidrug resistant bacteria [79]. Synthesized squalamine mimics were able to improve antibacterial activity of rifampin on various Gram negative bacteria, the effect was pronounced against *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Serratia marescens*, Fractional inhibitory concentration (FIC) was included between 0.046 to 0.5 [71]. It is noteworthy that other polyamines such as naphtylacetylspermine have been reported to permeabilize Gram negative bacteria increasing the permeability of the outer membrane and enhancing the activity of novobiocin and erythromycin. Addition of naphtylacetylspermine which was inactive against *E. coli* (MICs > 128 mg/L) at 8 mg/l lead to a reduction of the MICs value of novobiocin (128 mg/L) and erythromycin (64 mg/L) to 16 and 32 mg/L, respectively [78].

3- Other compounds

Antimicrobial activities of mono and bis acyl-polyamines are evaluated against *S. aureus* (MIC 3.9 to 250 µM) and *E. coli* (MIC 31.25 to 62.5 µM) reference strains. Some of them possess significant antimicrobial activity via permeabilization mechanism, two bis acyl polyamines sensitize effect to hydrophobic antibiotics (rifampin) by a factor 4.096, it was demonstrated that acyl chain length plays a role on membrane permeabilization. Activity of one compound among these family was measured in presence of physiological concentration of human serum albumin (677 µM) and shows a fourfold attenuation of MICs, for example on *E. coli* MICs 31.25 to 125 µM and on *S. aureus* MICs 3.9 to 15.6 µM, authors conclude that these acylpolyamines was attractive as an adjunct in antimicrobial therapy [80].

Dexamethasone spermine **32** and disubstituted dexamethasone spermine **33** (Figure 18) have been evaluated against *E. coli*. **33** with a good antibacterial activity and bacterial killing was observed at 5 µM. On the other hand, similar activities were observed with **33** against *B. subtilis* and *P. aeruginosa* whereas activity of **33** was reduced against *E. coli* in the presence of purified LPS, revealing interaction between LPS and **33** [81]. **33** has been also applied against cystic fibrosis clinical isolates such as *S. aureus*, with MICs ranging from 1.56 to 3.12 µM. Furthermore, it is noteworthy that **33** is able to enhance antibacterial activity of amoxicillin and clavulanic acid, tetracycline and amikacin against *S. aureus* suggesting a possible

synergistic effect of **33** with these antibiotics. Finally, **33** possess activity against biofilm of *P. aeruginosa*, at 25 µM **33** is able to decrease 50 % of biofilm mass [82].

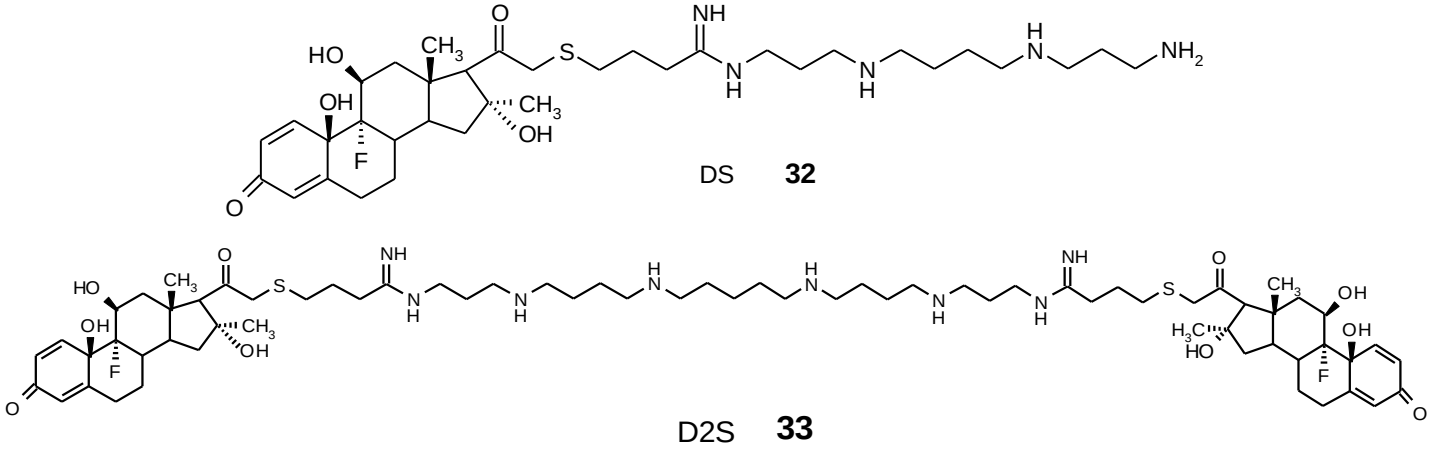

Fig. (18). Structure of DS, D2S and a synthetic mimic antimicrobial peptide.

Transfection properties

Cationic steroid antibiotic (CPAs) which possesses amphiphilic structure can be associated with a zwiterrion lipid dioleoyl-phophatidylethanolamine (DOPE) allowing transfection by facilitating the uptake of a plasmid in cell lines. It is noteworthy that the study of toxicity MTT demonstrates that CSA1/DOPE/DNA is slightly cytotoxic with 98% of viability compared to non treated cells [83].

Disinfectant properties

Antiseptics and disinfectants are widely used in hospitals in topical application and materials disinfection for the prevention of nososcomial infection [84]. Thus, medical devices with long residence times such as catheter were susceptible to bacterial biofilm colonization and may be a source of infection. Decontamination of devices was an approach to eradicate biofilm. Pollard et *al.* studied *in*

vitro the potential use of ceragenins in removing established biofilms in a model of a catheter lock solution. The authors found that CSA1 was able to eliminate biofilms of Gram positive and Gram negative bacteria at similar concentration with ciprofloxacin [85]. This compound shows also an activity against planktonic *P. aeruginosa* Xen 5 and biofilm formed by *H. pylori*, *S. aureus* and *E. faecalis.* Addition of 10 to 30 µM of CSA 1 induce a decrease of luminescence, which measure antibacterial activity, against *P. aeruginosa* Xen 5 of this compound, at 66, 93, 96, 98 and 99% in plasma, ascites, cerebrospinal fluid, saliva and brochoalveolar lavage, respectively [86]. Aminosterol derivatives which were previously described to possess a broad spectrum activity have been applied for a topical use. Thus, squalamine topical application was successfully realized in a *S. aureus* skin decolonization mouse model demonstrating that squalamine was able to reduce significantly and more rapidly *S. aureus* cutaneous colonization in comparison with classically used antibiotics such as mupirocin and fusidic acid [87].

Conclusion

In conclusion, it clearly appears that polyamines are essential for life and are widely involved for growth cells, cellular reparation, gene transcription, proteins and nucleic acids synthesis. On the other hand, due to their particular structure, polyamino derivatives open the way to the development of a new class of antimicrobial agents against MDR pathogens and could become a last-line therapeutic drug in the coming fifty years.

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Chapitre II: Partie 1

Synthèse et évaluation des activités antimicrobiennes de nouveaux dérivés polyaminostéroïdiens.

- **Synthesis of new 3,20-bispolyaminosterol squalamine analogues and evaluation of their antimicrobial activities.**

  Lamia Djouhri-Bouktab, Nicolas Vidal, Jean-Marc Rolain and Jean-Michel Brunel*

  Journal of Medicinal Chemistry, under press

- ***In vitro* activity of aminosterols against yeasts involved in blood stream infections.**

  Kamel Alhanout, Lamia Djouhri, Nicolas Vidal, Jean-Michel Brunel, Renaud Piarroux, Stephane Ranque *

  Medical Mycology, 2011, 49, 121-125.

Article 2

Synthesis of new 3,20-bispolyaminosterol squalamine analogues and evaluation of their antimicrobial activities.

Lamia Djouhri-Bouktab[1], Nicolas Vidal[2], Jean-Marc Rolain[1] and Jean-Michel Brunel[1*]

Journal of Medicinal Chemistry, **under press**

Résumé de l'article

L'émergence de souches bactériennes résistantes aux antibiotiques constitue un problème de santé publique d'où la nécessité de la recherche de nouvelles molécules à activité antimicrobienne. La membrane bactérienne est la cible de nombreux antibiotiques actifs sur les bactéries Gram positive et Gram négative. La membrane des bactéries forme une barrière contre de nombreux antibiotiques et protéases. De nombreux dérivés sont actifs sur les bactéries Gram positive mais moins actifs sur les bactéries Gram négative. La perméabilité membranaire chez les bactéries Gram négative est due à la liaison formée entre le lipide A et les cations divalents (Ca^{2+} et Mg^{2+}). Les dérivés susceptibles d'attaquer les sites de liaisons peuvent perturber l'intégrité membranaire comme les peptides cationiques et les polyamines, les polyamines sont ainsi capables d'augmenter la sensibilité des bactéries Gram négative aux antibiotiques hydrophobes tels que l'érythromycine.

En 1998, deux alcaloïdes cationiques ont été isolés, un saligcinnamide et la N-metyl epipachysamine-D à partir d'un arbuste *Sarcococca saligna* qui se sont révélés être très actifs sur de nombreux pathogènes humains. Dans le même laps de temps, la squalamine et la trodusquemine ont été identifiées chez le requin, *Squalus acanthias* et possèdent quant à eux de très bonnes activités sur des souches bactériennes multirésistantes. De nombreux analogues de la squalamine ont été synthétisés au laboratoire mettant en œuvre une méthode d'amination réductrice au titane original. Il a ainsi pu être

démontré que la nature et la taille de la chaîne polyaminée ont un effet déterminant et non négligeable dans les activités antimicrobiennes de cette famille de molécules.

Dans ce travail, nous avons réalisé la synthèse d'une nouvelle classe de 3,20 bis(polyaminostérols) analogues de la squalamine et de la trodusquemine grâce à la méthode d'amination réductrice au titane en une seule étape. Les rendements de synthèse varient de 18 à 82 % avec une diastéréoselectivité supérieure à 95% dans la majorité des cas. Nous avons évalué leur activité antibactérienne sur un large panel de souches bactériennes de référence ainsi que des isolats cliniques multirésistantes. Les CMIs varient entre 2.5 et 20 µg/mL sur les *Staphylococcus aureus* oxacilline sensible et résistant.

En ce qui concerne les bactéries Gram négative, les activités antimicrobiennes sont très variables en fonction de la bactérie testée, cette famille de composés est active sur les souches d'*Escherichia coli* et *Pseudomonas aeruginosa,* les CMIs varient de 2.5 à 40 µg/mL. Ces composés sont moins actifs sur les souches résistantes telles que *Burkholderia cepacia et Iquilinus limosus,* les CMIs varient de 20 à > 40 µg/mL. Une corrélation entre la longueur de la chaine polyaminée portée par le stérol et les activités antimicrobiennes a été observée chez *E. coli.* Ces résultats suggèrent que la nature de la chaine polyaminée portée par le stérol joue un rôle essentiel dans les activités biologiques de ces dérivés. Nous avons également démontré que le mécanisme d'action est différent vis-à-vis des bactéries Gram positive et Gram négative. En effet, l'addition de sels divalents à

10 mM tels que Ca^{2+} et Mg^{2+} induit une augmentation de la CMI de la squalamine, de la colistine et du dérivé DAS-1 sur *E. coli*, alors que l'activité de la tobramycine n'est pas affectée. Dans le cas des bactéries Gram positive, l'ajout des sels divalents n'a eu en revanche aucun effet sur les CMIs de la squalamine, de la colistine et de la tobramycine. L'étude de survie montre un effet bactéricide pour la squalamine et un dérivé bis(polyaminostéroïdien), ces deux composés sont aussi capables de réduire de 5 Log le nombre de bactéries viables des espèces *S. aureus* et *P. aeruginosa* après 1 h et 2 h d'incubation, respectivement. L'action de la squalamine et du dérivé bis(polyaminostéroïdien) a été étudiée par la mesure de la cinétique d'efflux d'ATP qui est un indicateur de la présence de lésions membranaires pendant 20 min, révélée par la méthode de bioluminescence. La squalamine induit un relargage rapide d'ATP chez *S. aureus*, l'efflux maximal (100%) est observé au bout de 5 min, alors que dans chez *E. coli* le relargage d'ATP est temps dépendant. Par ailleurs, on observe une augmentation de la fluorescence au bout de 3 min chez les *S. aureus* traité par la squalamine et le dérivé bis(polyaminostéroïdien) indiquant une dépolarisation de la membrane bactérienne, le dérivé bis(polyaminostéroïdien) induit un effet dépolarisant beaucoup plus important que la squalamine, probablement due au nombre de charges positives moins élevé chez cette dernière. A l'inverse, aucun effet dépolarisant n'a été observé chez les bactéries Gram négative telle qu'*E. coli* quelque soit le composé mis en œuvre. L'inhibition des activités antibactériennes lors

de l’ajout des sels divalents suggèrent que ces dérivés interagissent avec les charges négatives des groupements phosphates des lipopolysaccharides de la membrane des bactéries Gram négative. Ce mécanisme d’action original réduit la probabilité d’émergence des bactéries résistantes aux dérivés aminostéroïdiens.

En conclusion, des études restent également à mener afin de déterminer la cytotoxicité de ces dérivés bis(polyaminostéroïdiens) pour envisager leur utilisation potentielle en thérapeutique humaine.

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BRIEF ARTICLE

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Synthesis of New 3,20-Bispolyaminosteroid Squalamine Analogues and Evaluation of Their Antimicrobial Activities

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Supporting Information

ABSTRACT: 3,20-Amino- and polyaminosteroid analogues of squalamine and trodusquemine were synthesized involving a stereoselective titanium reductive amination reaction in high chemical yields in numerous cases. These derivatives were evaluated for their in vitro antimicrobial properties against references and clinical bacterial strains exhibiting minimum inhibitory concentrations of 2.5−40 μg/mL. The mechanism of action of these derivatives was determined using bioluminescence for ATP efflux measurements and fluorescence methods for membrane depolarization assays.

INTRODUCTION

Since their massive successful use for treatment of numerous bacterial infections in the past century, chemical antibiotics appeared as one of the great health successes, decreasing morbidity and mortality. However, this has led to high levels of unsuitable prescribing, contributing to recent rises in numbers of antibiotic-resistant bacteria. Thus, emergence of multidrug resistant (MDR) microorganisms such as methicillin resistant *S. aureus* and vancomycin resistant *Enterococcus* or *Pseudomonas* has prompted efforts to develop new classes of antibiotics.[1−3] Bacterial membrane structure constitutes an appealing target, since the latter is highly conserved among most species of both Gram-negative and Gram-positive bacteria. Resistance to membrane active antibiotics requires major changes in membrane structure that in turn influences the permeability barrier, increasing susceptibility to hydrophobic antibiotics. Furthermore, the outer membrane of Gram-negative bacteria forms an effective barrier to proteases, lysozymes, and many types of antibiotics.[4] Consequently, numerous molecules that are active against Gram-positive organisms are much less active against Gram-negative bacteria and vice versa. Most of the responses to Gram-negative bacteria could be attributed to lipopolysaccharides (LPS) and their lipid A anchor, which are a primary component of the outer membrane.[5−10] It is widely held that the permeability barrier of the outer membrane is formed via cross-bridging between lipid A molecules and divalent cations (Ca^{2+} and Mg^{2+}).[11] Thus, it is well-known that metal ion chelators such as EDTA, certain cationic peptides,[12] and polyamines, which can attack the binding sites of divalent cations, are able to disrupt the organization of the outer membrane, increasing its permeability and therefore sensitizing bacteria to hydrophobic antibiotics. In this context, an attractive approach for the development of antibacterial agents is the use of compounds targeting membranes of Gram-negative bacteria, since they are not expected to readily induce resistance formation. Among all the numerous antibiotics developed to date, few compounds possessing a cationic steroid structure have been identified and studied. Thus, in 1998 two new cationic pregnane-type steroidal alkaloids, saligcinnamide **1** and pachysamine H **2**, were isolated from the EtOH extracts of the roots and stems of *Sarcococca saligna* and these compounds exhibited good antibacterial activity against several human pathogenic bacteria (Figure 1).[13,14] Among all the envisioned strategies in our laboratory in the search for novel host defense agents, squalamine **3a** and trodusquemine **3b** (Figure 1) were identified as the first natural aminosterols from the dogfish shark, *Squalus acanthias*, exhibiting potent antimicrobial activities against MDR bacterial strains.[15,16] On the basis of such results, we have recently reported the design of new aminosteroid derivatives easily obtained from cheap and available precursors through an original titanium reductive amination reaction.[17,18] It has also clearly appeared that the presence of a polyamino moiety is crucial to encounter high antimicrobial activities.[19,20] In the our work, we report herein the design and antimicrobial activities of a new class of bis-(polyamino)steroid derivatives. We will also report a deeper analysis of their mechanism of action against Gram-negative and Gram-positive bacteria, pointing out the original mechanism of action of this class of derivatives.

RESULTS AND DISCUSSION

Using an efficient titanium reductive amination developed in our laboratory, we have envisioned a one step procedure for the preparation of new bis(polyamino)steroid derivatives from progesterone available in large amounts according to the following synthetic pathway (Table 1). First, it clearly appears that isolated yields of **4c** are highly solvent dependent. Thus, the expected amino derivative **4c** was obtained in 63% yield, performing the reaction in MeOH (Table 1, entry 1) whereas only moderate yields of 30%, 19%, and 33% were encountered performing the reaction in CH_2Cl_2, toluene, and THF, respectively (Table 1, entries 3−5). The influence of the nature of the titanium source involved was also investigated, and chemical yield variations of 19−63% were obtained (Table 1, entries 1, 6−8), the best result having been observed using Ti(O-*i*-Pr)$_4$ as titanium source.

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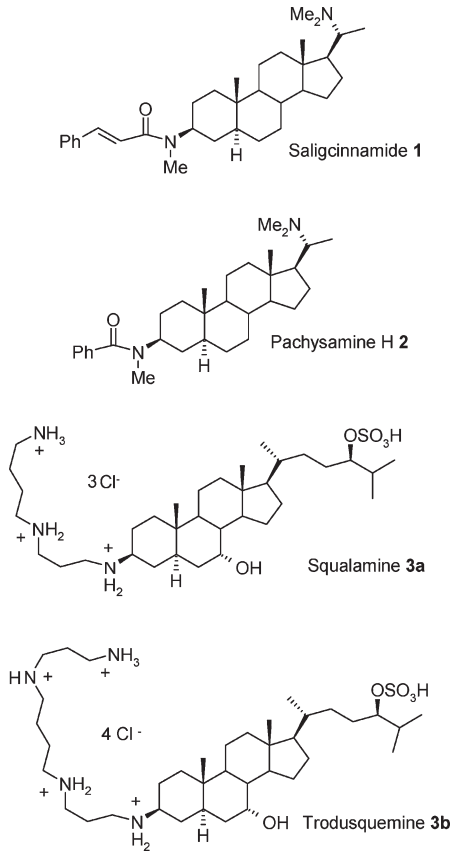


Figure 1. Structures of saligcinnamide **1**, pachysamine-H **2**, squalamine **3a**, and trodusquemine **3b**.

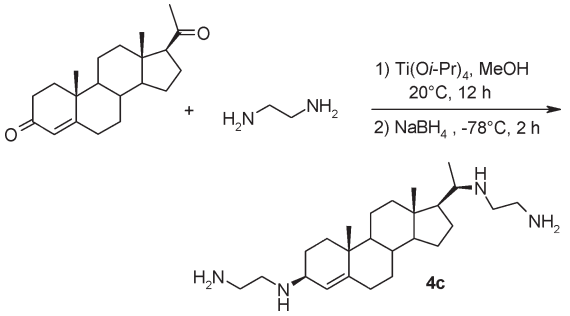
Furthermore, under these best experimental conditions, increasing the reaction temperature from −78 to 0 °C led to a significant decrease of the diastereoselectivity from 95% to 60% de, respectively (Table 1, entries 1 and 2).

A mechanistic rationale, including a nucleophilic attack of the amino group to a carbonyl compound activated by a Lewis acid and a transient imine species that can be subsequently reduced, is proposed. A transition state model has been also rationalized to account for the stereoinduction in the reaction leading exclusively to the formation of the β-amino or bis(polyamino) derivative due to steric hydrogen hindrance, suggesting that the hydride attack occurs at the C-3 carbon or C-20 carbon in an α position generating principally the 3β,20β-aminocholestane derivative **4**. Thus, this transition state allows us to justify that the formation of the 3α,20α parent derivative is unfavored at low temperature.[21−23]

On the other hand, whatever the nature of the considered diamine or polyamine was, the expected products were obtained in chemical overall yields of 18−82% and excellent diastereoselectivities of up to 95% de in all cases (Table 2).

Chronic microbial colonization of the respiratory tract, leading to exacerbations of pulmonary infection, constitutes the major cause of disease and death in patients with cystic fibrosis (CF). Typical pathogens in respiratory secretions of CF patients include Gram-positive and Gram-negative such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Inquilinus limosus*, and *Burkholderia cepacia*.[24] In the context of our studies, all of the synthesized compounds were screened for their antimicrobial activities against Gram-positive and Gram-negative bacteria strains and found to possess activities against the microorganisms listed in Table 3. Most of the compounds (typically derivatives **4c**−**l**) present excellent activities against Gram-positive bacteria, exhibiting similar MIC values of 2.5−10 μg/mL against oxacillin sensitive or resistant *Staphylococcus aureus* strains except for **4a** and **4b**, suggesting that the presence of polyamino chains is necessary to lead to biologically active compounds. On the other hand, all the derivatives possess moderate to excellent antimicrobial activities against Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* strains with MIC values of 2.5−40 μg/mL whereas low activities were encountered against *Inquilinus limosus* and *Burkholderia cepacia* strains, suggesting that the structure and the nature of the polyamino groups attached to the steroid moiety play an important role in the mechanism of action. This assumption is supported by the correlation observed between the number of carbons constituting the polyamino chain attached to the steroid core and the antimicrobial activity encountered against *P. aeruginosa* (Figure 2B). Surprisingly, no similar effect was observed against *S. aureus* Gram-positive bacteria, since the antimicrobial activity remained unchanged whatever the derivative used, suggesting that the mechanism of action of these compounds is different depending on the class of bacteria considered (Figure 2A). As shown in Table 4, the addition of Mg^{2+} at 10 mM final concentration increased the MIC of colistin, squalamine, and **4i** against *E. coli* at least 4 times, whereas the MICs of tobramycin were not affected. Moreover, MICs of squalamine, colistin, or tobramycin remain unchanged by Mg^{2+} supplementation in the case of *S. aureus*. Furthermore, **4i** exhibited a complete killing of *P. aeruginosa* reference strains in 2 h (Figure 3A). Moreover, **4i** showed a direct bactericidal effect against *S. aureus* reference strain reflect by nearly a 5 log decrease

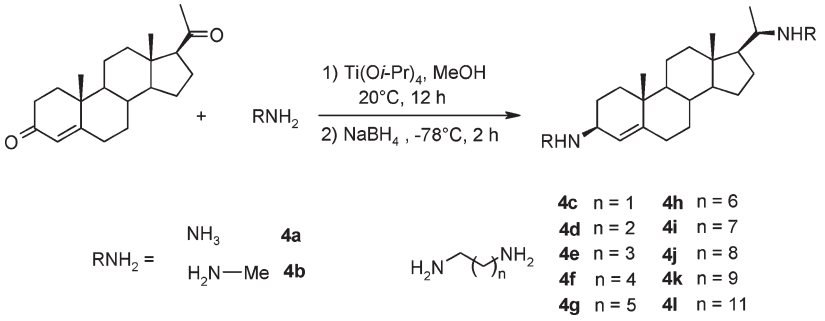
Table 1. Titanium(IV) Reductive Amination Reaction of Progesterone with 1,2-Diaminoethane under Various Experimental Conditions



entry	titanium source	solvent	yield (%)[c]	de (%)[d]
1[a]	Ti(O-*i*-Pr)$_4$	MeOH	63	>95
2[b]	Ti(O-*i*-Pr)$_4$	MeOH	72	60
3[a]	Ti(O-*i*-Pr)$_4$	CH_2Cl_2	30	91
4[a]	Ti(O-*i*-Pr)$_4$	toluene	19	92
5[a]	Ti(O-*i*-Pr)$_4$	THF	33	>95
6[a]	Ti(OEt)$_4$	MeOH	41	>95
7[a]	Ti(OBu)$_4$	MeOH	30	>95
8[a]	Ti(O-*t*-Bu)$_4$	MeOH	19	

[a] Reaction performed at −78 °C for 12 h in MeOH on a 0.39 mmol scale of progesterone, Ti(O-*i*-Pr)$_4$ (2.02 mmol), and the amine (6 mmol). [b] Reaction performed at 0 °C. [c] Isolated overall yield. [d] Diastereomeric excess (de) was evaluated by HPLC and ^{1}H and ^{13}C NMR.

Table 2. Titanium(IV) Reductive Amination Reaction of Progesterone with Various Diamines



entry[a]	product	amine	yield (%)[b]
1	**4a**	ammonia	18
2	**4b**	methylamine	37
3	**4c**	1,2-diaminoethane	63
4	**4d**	1,3-diaminopropane	82
5	**4e**	putrescine	73
6	**4f**	cadaverine	61
7	**4g**	1,6-diaminohexane	45
8	**4h**	1,7-diaminoheptane	53
9	**4i**	1,8-diaminooctane	56
10	**4j**	1,9-diaminononane	64
11	**4k**	1,10-diaminodecane	46
12	**4l**	1,12-diaminododecane	29

[a] Reaction performed at −78 °C for 12 h in MeOH on a 0.39 mmol scale of progesterone, Ti(O-*i*-Pr)$_4$ (2.02 mmol), and the amine (6 mmol). [b] Isolated overall yield.

Table 3. Antimicrobial Activities of 3,20-Bispolyaminosteroid Derivatives 4a−k[a]

	minimum inhibition concentration (MIC (μg/mL))							
	Gram-positive bacteria			Gram-negative bacteria				
	S. aureus			*E. coli*	*P. aeruginosa*	*P. aeruginosa*	*I. limosus*	*B. cepacia*
compd	ref, 2	Oxa S, 10	Oxa R, 6	ref, 1	ref, 2	CS S, 12	CS R, 3	CS R, 2
squalamine	1.25	0.3	1.25	5	2.5	5−20	5	>40
TOB	1.25	1.25		2.5	5	5−20	>40	>40
CS				2.5	5	5−20	>100	>40
4a	40	>40	40	>40	>40	>40	>40	>40
4b	40	20	20	>40	>40	>40	>40	>40
4c	20	20	10−20	20	20	20−40	>40	>40
4d	10	10	5−10	10	10	20−40	20−40	>40
4e	5	10	5−10	10	20	20−30	40−80	>40
4f	5	5−20	10−20	10	10	20−80	40−80	>40
4g	2.5	5−10	5	5	10	20−40	20	>40
4h	2.5	2.5−5	5−10	5	10	10−30	20−40	>40
4i	2.5	2.5	5	5	2.5	2.5−10	10−20	>40
4j	5	5−10	5	20	40	40−80	20−40	>40
4k	10	5−10	5	20	40	40−80	20−40	>40
4l	10	5−10	10	20	40	40−80	20−40	>40

[a] ref: reference strain. CS: colistin. Oxa: oxacillin. S: sensitive. R: resistant;.TOB: tobramycin.

in the counts of this bacteria by 1 h whereas 2 h are necessary using tobramycin (Figure 3B). On the other hand, **4i** and squalamine effects on bacterial membrane integrity were investigated by measuring intracellular ATP efflux kinetics during 20 min. For *E. coli*,

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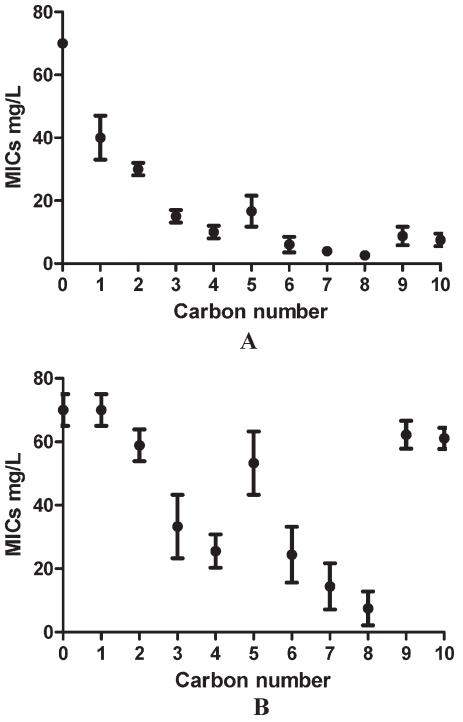

Figure 2. Correlation of the 3,20-bispolyaminosteroid derivatives activities with respect to the length of the polyamino chain against *S. aureus* (A) and *P. aeruginosa* strains (B).

Table 4. Effect of Divalent Cation Salt on Antibacterial Activity of Colistin, Tobramycin, Squalamine, and Derivative 4i against *E. coli* and *S. aureus*[a]

	minimum inhibition concentration (MIC (μg/mL))							
	without Mg^{2+}				with Mg^{2+} (10 mM)			
strain	squalamine	CS	TOB	**4i**	squalamine	CS	TOB	**4i**
E. coli ATCC 25922	2,5	0.5	2	2,5	32	16	1	10
S. aureus ATCC 25923	1,25	>128	1	2.5	1.25	>128	2	5

[a] CS: colistin. TOB: tobramycin

a time-dependent ATP release was observed with less than 35% of maximal efflux (Figure 4A). Conversely, squalamine incited a rapid ATP release from *S. aureus* reaching 100% of maximal efflux after only 5 min (Figure 4B). In this context, treatment with colistin resulted in a slight but significant ATP release ($P < 0.0001$) in the case of *E. coli* leading to 4−5% of maximal efflux after 5 min while no significant effect was noticed in the case of *S. aureus* all along the test time ($P < 0.0001$, Figure 4). Finally, no depolarizing effect was noticed in squalamine-treated *E. coli* (Figure 5A), whereas in the same conditions, squalamine prompted a flagrant depolarization of *S. aureus* bacterial membrane depicted by a rapid and strong increase in relative fluorescence units ΔRFU reaching in less than 3 min 80% of maximal RFU (Figure 5B). On the other hand, **4i** appears to be a 4 times better depolarizing agent than squalamine in both cases because of the presence of a high number of positive charges.

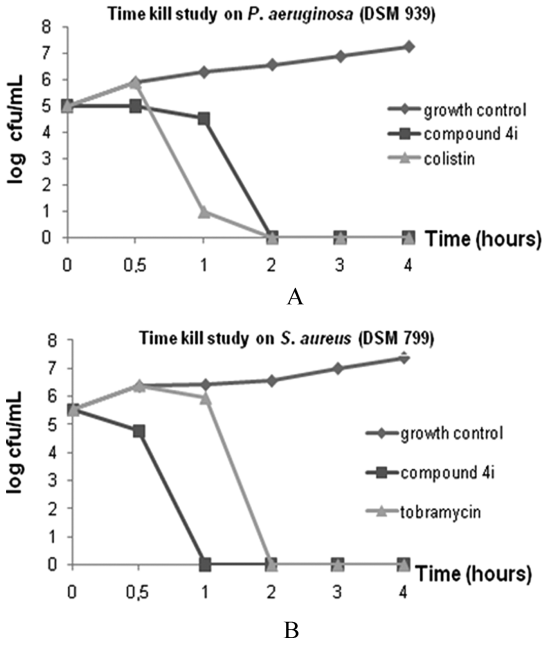

Figure 3. Time-kill curves of derivative **4i** at 1× the MIC over a 4 h period against *P. aeruginosa* (DSM 939) (A) and *S. aureus* (DSM 799) (B) strains.

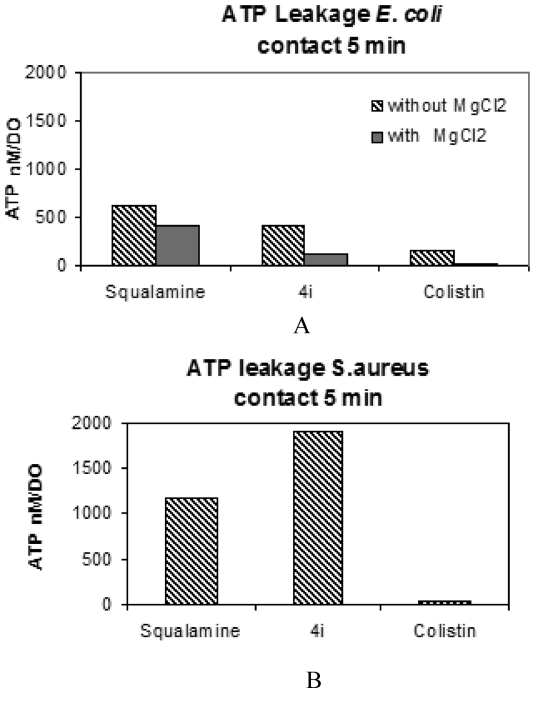

Figure 4. Effect of compound **4i** on ATP efflux for treated *E. coli* (A) and *S. aureus* (B) bacteria.

A potent mechanism of action had been previously described with the polymyxin antibiotic colistin that, by using positive amine groups, interacts with the negative phosphate groups of LPS displacing divalent cations such as Ca^{2+} and Mg^{2+}.[10,11] Accordingly, an inhibitory effect of Ca^{2+} and Mg^{2+} on colistin but also on bispolyaminosteroid derivatives activities against *E. coli* was found indicating that the interaction with the negatively

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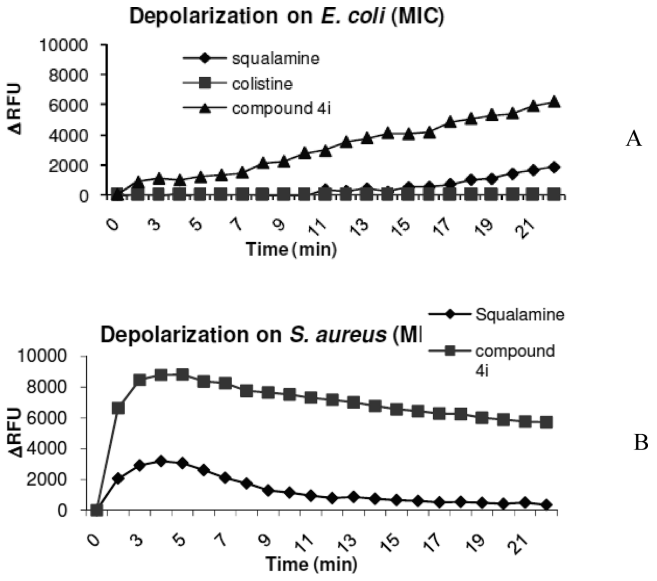

Figure 5. Depolarization of bacterial membrane of *E. coli* (A) and *S. aureus* (B) bacteria in the presence of squalamine and compound **4i**.

charged phosphate groups in LPS structure is mandatory for both agents to be active. Interestingly, **4i** showed markedly faster killing rate than colistin against Gram-negative bacteria, suggesting that both compounds might interact differently with bacterial membranes. Accordingly, though the availability of negatively charged phosphate groups represents a common requirement for the activity of **4i** and colistin, both agents probably exhibit different series of action soon after interaction with LPS. Indeed, by use of intracellular ATP efflux as an indicator of membrane lesions, treatment of *E. coli* with **4i** resulted in significantly higher and faster ATP efflux compared to colistin and squalamine, suggesting that LPS damage induced by derivative **4i** is clearly greater and faster than that caused by colistin and squalamine. However, this would not be valid for Gram-positive bacteria that are devoid of LPSs. Thus, it was not surprising that the divalent cations had no effect on **4i**, squalamine, colistin, or tobramycin activities against the Gram-positive bacterium *S. aureus*. Remarkably, derivative **4i** demonstrated a faster killing rate against *S. aureus* than that noted with Gram-negative bacteria, signifying that this compound may possess a rapid and direct bactericidal effect against Gram-positive bacteria. Moreover, **4i** produced an instantaneous intracellular ATP efflux in the case of *S. aureus*, indicating that a rapid phenomenon might specifically be involved in **4i** mode of action against Gram-positive bacteria. Indeed, **4i** led to a strong depolarization of *S. aureus* membranes while a weak depolarization was observed for tested Gram-negative bacteria. Thus, **4i** and by extension other related amino steroids have a particular mode of action mediated by bacterial membrane disruption reducing the possibility of resistance emergence. Altogether and without excluding other intra- or extracellular targets of the action of **4i**, our results indicate that this family of compound acts by disrupting the outer membrane of Gram-negative bacteria in a detergent-like mechanism of action and by depolarizing bacterial membrane of Gram-positive bacteria.

CONCLUSION

Because of the unique mechanism of action, current studies are now underway to evaluate the potentiality of such derivatives by determining their cytotoxicity and their potent use mainly as disinfectant, detergents, or topical antimicrobial agents.

EXPERIMENTAL SECTION

The purity of the compounds was checked by analytical HPLC (C18 column, MeOH/CH_3CN) with PDA detector spanning from 210 to 310 nm. All compounds were >95% pure, as determined by analytical HPLC-PDA at 214 and 254 nm.

General Procedure for the Titanium-Mediated Reductive Amination Reaction of 4c. A mixture of progesterone (123 mg, 0.39 mmol), titanium(IV) isopropoxide (573 μL, 2.02 mmol), and 1,8-diaminooctane (6 mmol) in absolute methanol (5 mL) was stirred under argon at room temperature for 12 h. Sodium borohydride (38 mg, 1 mmol) was then added at −78 °C, and the resulting mixture was stirred for an additional 2 h. The reaction was then quenched by adding water (1 mL), and stirring was maintained at room temperature for 20 min. The resulting inorganic precipitate was filtered off over a pad of Celite and washed with methanol and ethyl acetate. The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo to afford the expected crude amino derivative which was purified by flash chromatography, affording the expected amino derivative. Purification by column chromatography (silica gel, CH_2Cl_2/MeOH/NH_4OH (32%), 7:3:1) afforded a pale yellow pasty solid in 56% yield. ^{1}H NMR (300 MHz, CD_3OD): δ 5.22 (s, 1H), 3.44−3.22 (m, 4H), 2.29−1.02 (m, 60H), 0.82−0.76 (m, 5H). ^{13}C NMR (75 MHz, CD_3OD): δ 148.49, 128.22, 59.83, 57.59, 56.24, 48.03, 47.98, 44.17, 42.92, 41.21, 38.55, 34.06, 31.84, 30.97, 28.94, 28.39, 27.32, 25.99, 22.62, 18.95, 13.20. $C_{37}H_{70}N_4$ MS (ESI$^+$) m/z 571.5634 (100%, $[M + H]^+$).

ASSOCIATED CONTENT

Supporting Information. Details for synthetic preparations, analytical data of all compounds, and biological test systems. This material is available free of charge via the Internet at http://pubs.acs.org.

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Article 3

In vitro activity of aminosterols against yeasts involved in blood stream infections

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Medical Mycology, 2011, Feb; 49 (2): 121-5

Résumé de l'article

Les infections fongiques sont responsables de nombreuses complications particulièrement chez les patients atteints de mucoviscidose d'où la nécessité de la recherche de nouvelles molécules à activités antifongiques. La squalamine et les dérivés aminostéroïdiens possèdent de bonnes activités antimicrobiennes démontrées sur diverses souches cliniques de bactéries et sur certaines souches de levures de référence.

Dans ce travail, nous avons évalué les activités antifongiques *in vitro* de la squalamine, d'un aminostérol DAS-1 et de nombreux antifongiques tels que l'amphotericine B et la caspofongine sur des souches de référence et 21 isolats cliniques de levures impliquées dans de nombreuses fongémies. Les CMIs varient de 8 à 16 µg/mL pour la squalamine et de 1 à 2 µg/mL pour le dérivé DAS-1. La toxicité a été évaluée par la mesure de la concentration hémolytique (IC_{50}) *in vitro* en utilisant des globules rouges humains. Les IC_{50} de la squalamine, DAS-1 et l'amphotéricine B sont de 90, 40 et supérieure à 100 mg/L, respectivement. Les IC_{50} sont largement supérieures aux activités antifongiques avec un rapport IC_{50}/CMIs de 8 et 30 pour la squalamine et DAS-1, respectivement, suggérant un ratio toxicité/activité acceptable. Nous avons aussi mesuré l'activité fongicide en réalisant un "time kill study". Le dérivé DAS-1 ainsi que l'amphotéricine B présente le même effet fongicide observé au bout de 4 h d'incubation alors que dans le cas de la squalamine celui-ci a

nécessité un temps plus long (8h). L'efflux d'ATP intracellulaire mesuré aux CMIs de ces composés démontre un relargage progressif d'ATP en fonction du temps pour la squalamine et le dérivé DAS-1, le maximum est observé au bout de 40 min d'incubation alors qu'on note une absence de libération d'ATP pour l'amphotericine B. L'ensemble des résultats obtenus suggère que les dérivés aminosteroïdiens agiraient par déstabilisation de la structure membranaire des levures comme les détergents. Ces résultats préliminaires méritent d'être approfondie par la mesure de la toxicité et de l'activité *in vivo* sur un modèle animal afin d'envisager leur utilisation potentielle en tant qu'agents antifongiques.

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Original Articles

In vitro activity of aminosterols against yeasts involved in blood stream infections

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Squalamine and other aminosterols have demonstrated interesting antimicrobial activities against clinical bacterial isolates and a limited number of reference yeast strains. We aimed to test whether squalamine and a synthetic aminosterol derivative (ASD) display any *in vitro* activity comparable to currently available systemic antifungals, an acceptable safety index, as well as to provide insights into their mechanism of action. The minimum inhibitory concentrations (MICs) of squalamine, ASD and available antifungals were determined against 21 yeast isolates that were recovered from cases of fungemia. Remarkably, homogeneous MICs ranging from 8–16 mg/L and from 1–2 mg/L were noted for squalamine and ASD, respectively, as opposes the heterogenous *in vitro* activity of available systemic antifungals. Aminosterols induced haemolysis, a surrogate for toxic effects to mammalian cells, at concentrations high above their MICs. In time-kill studies, killing was as fast with ASD as with amphotericin B. Both aminosterols induced a time-dependent disruption of yeast membrane, as evidenced by gradual increase of ATP efflux. In conclusion, our preliminary data indicate that aminosterols have the potential to be further developed as antifungals. Additional work is warranted to assess their toxicity and activity in experimental models.

Keywords squalamine, antifungal, resistance, therapy

Introduction

Squalamine (Fig. 1) is a natural aminosterol first isolated from the tissues of the dogfish shark *Squalus acanthias*, which possesses numerous therapeutic values such as anti-angiogenic and antimicrobial properties [1]. The synthesis of squalamine is known to be sophisticated and expensive, while many squalamine-related compounds, namely aminosterol derivatives, have been synthesized from easily available and less expensive starting materials. The latter have also demonstrated interesting antimicrobial activities [2]. A phase II study evaluating the antiangiogenic effect of squalamine demonstrated that this aminosterol compound was safely administered to cancer patients in a continuous 5-day infusion of 300 mg/m^2/day [3]. We have recently reported that aminosterol compounds possess interesting antibacterial properties, as determined with various bacterial clinical isolates containing multidrug-resistant genes [4]. It has been shown that squalamine acts by disrupting the outer membrane of Gram-negative bacteria, as reflected by its increased permeability to hydrophobic dyes and induced intracellular ATP efflux by squalamine [5]. Invasive yeast infections also represent a major threat, particularly for immunocompromised patients [6,7]. In this patient population, fungal infections are known to be associated with high morbidity and mortality, especially when the pathogens involved present natural or acquired resistance to available antifungal agents [7–9]. Antifungal activity of aminosterols has been reported in a limited number of yeast reference strains [10]. However, the question of whether aminosterol derivatives have the potential to be developed as clinically

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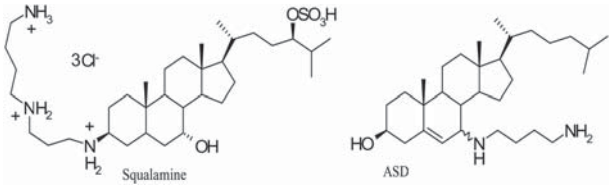


Fig. 1 Structures of squalamine and a synthesized aminosterol ASD.

useful antifungal agents needs to be addressed. Hence, this study aimed to compare the *in vitro* activity of squalamine and a synthetic aminosterol derivative (ASD, Fig. 1) to those of currently available systemic antifungals against reference and clinical yeast isolates through *in vitro* antifungal susceptibility testing and time-kill studies. Additionally, the *in vitro* toxicity of tested aminosterols and their effects on the yeast membrane were investigated.

Material and methods

Squalamine was a generous gift from Pr M. Zasloff (Georgetown University, Washington). ASD was synthesized as previously reported [10]. Stock solutions of squalamine were prepared in water and ASD in methanol. The antifungal agents tested were amphotericin B AMB) (Bristol Myers Squibb, Rueil-Malmaison, France), fluconazole (FLC) (Pfizer, Paris, France, itraconazole (ITC) (Cipharmed, Paris, France), voriconazole (VRC) (Pfizer, Paris, France) and caspofungin (CSN) (Merck Sharp & Dohme-Chibret, Paris, France).

The reference control strains used in this work were *Candida albicans* (ATCC 90028), *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019. A collection of 21 bloodstream yeast isolates were tested including; *C. albicans* ($n = 2$), *C. glabrata* ($n = 2$), *C. guilliermondii* ($n = 1$), *C. krusei* ($n = 3$), *C. lusitaniae* ($n = 3$), *C. parapsilosis* ($n = 5$), *C. tropicalis* ($n = 3$), and *Cryptococcus neoformans* ($n = 2$). The clinical isolates were cultured from blood samples from patients hospitalized at the University Hospital of Marseille (France) in Mycosis IC/F bottles (Becton Dickinson Diagnostics, France). Purity was checked on CHROM agar *Candida* chromogenic medium (Bio-Rad, France). Species identification was based on direct immunological tests (Bichrolatex Albicans and Krusei Color, Fumouze, France), microscopical morphology on PCB medium, carbohydrate assimilation profile using Auxacolor (Sanofi Diagnostic Pasteur, France) and, when necessary, ID 32C (Biomerieux, France). All identifications were confirmed by sequence analysis of the internal transcribed spacers (ITS) of the rDNA gene using a previously described procedure [11]. The ITS sequences of different fungi were aligned to each other and to sequences retrieved from the NCBI GenBank database, using multiple sequence alignment software ClustalX. A phylogenetic tree was constructed using the neighbour-joining clustering method implemented using MEGA software – version 4. The ITS sequence data of *Candida tropicalis* AB43708, *C. guilliermondii* FJ515181, *C. lusitaniae* EF136370, *Cryptococcus neoformans* FJ011544 and *Ornithonyssus bacoti* AM903318 available in the GenBank were added to the phylogenetic tree.

The *in vitro* minimum inhibitory concentrations (MICs) of squalamine, ASD and currently available antifungals indicated above were determined in duplicate by the broth microdilution method according to CLSI standard documents M27-A2 [12].

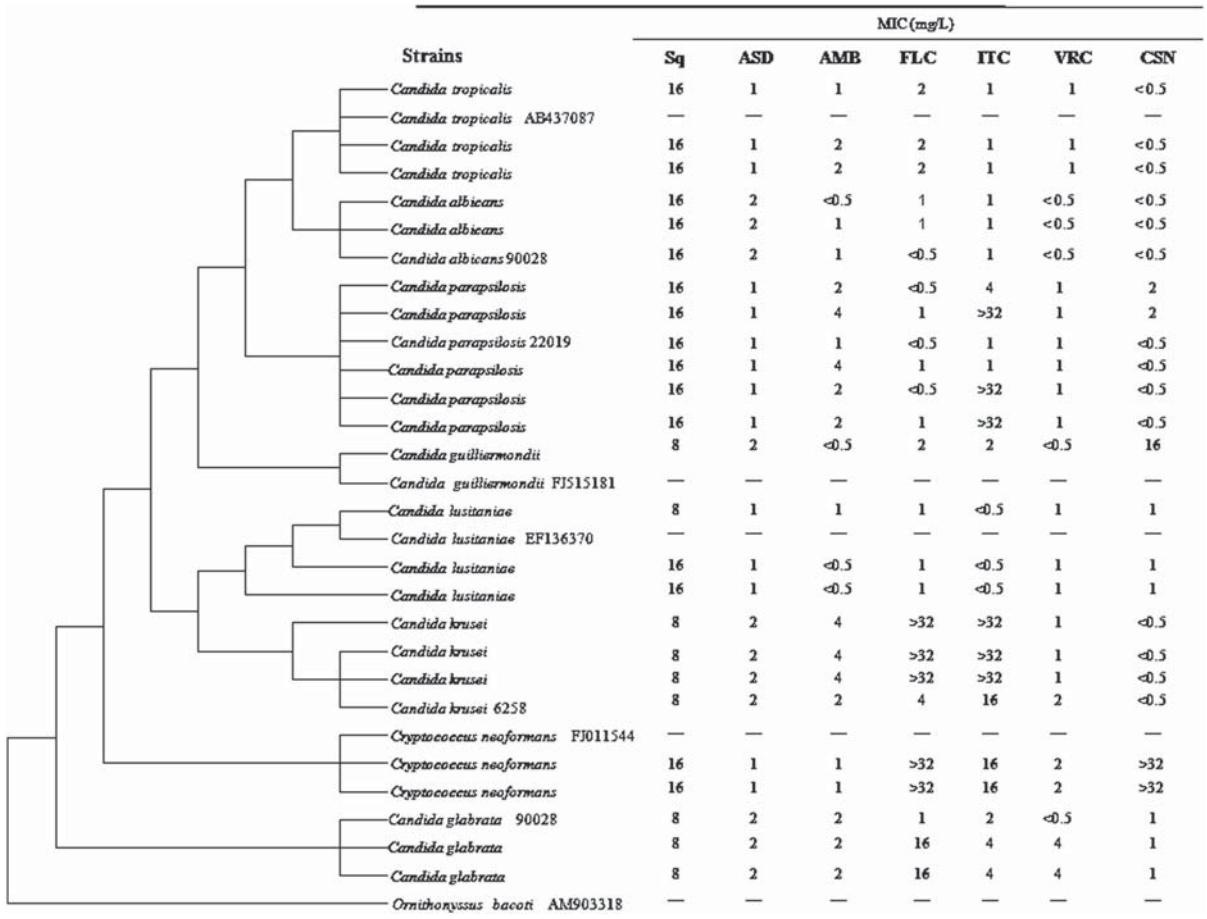
Possible toxic effects of squalamine and ASD on mammalian cells were investigated by measuring haemolysis of red blood cells, an accepted indicator of the *in vitro* toxicity of these compounds [13]. The haemolytic activities of squalamine, ASD, and amphotericin B were measured and the half maximal haemolytic concentration (IC_{50}) was determined as previously described [13]. Maximal haemolysis was considered to be produced by 50 μg/ml solution of cetyl trimethylammonium bromide (CTAB). Human red blood cells from fresh blood (haematocrit ~5%) were suspended in PBS and incubated for 1.5 h at 37°C after the addition of serial dilutions of the tested compounds. After centrifugation at 2000 *g*, relative haemoglobin concentration in supernatants was monitored by measuring the absorbance at 540 nm.

The time-kill assays were performed as previously described [14]. Two reference strains, *C. albicans* ATCC 90028 and *C. glabrata* ATCC 90030, were exposed to squalamine, ASD and amphotericin B (AMB) at concentrations equal to their MIC values and the killing rates were measured over time. A fungicidal effect was defined as a >99.9% reduction in the number of colony forming units/ml relative to the starting inoculum.

The kinetics of intracellular ATP efflux was determined as previously described [5]. Briefly, a suspension of *C. albicans* ATCC 90028 in Müller Hinton broth was added to solutions of 1 × MIC of squalamine, ASD, AMB and CTAB 5 μg/ml and incubated at 32°C. The ATP efflux kinetic was measured every 10 min for 60 min.

Results

The MICs of squalamine and ASD against reference yeast strains and a panel of clinical yeast isolates recovered from blood were compared to those of currently available systemic antifungals, including AMB, fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), and caspofungin (CAS) (Fig. 2). The MICs of currently used systemic antifungal drugs against control strains were within the expected ranges (Fig. 2). MICs for squalamine and ASD against tested isolates ranged from 8–16 mg/l and from



Strains	MIC (mg/L)						
	Sq	ASD	AMB	FLC	ITC	VRC	CSN
Candida tropicalis	16	1	1	2	1	1	<0.5
Candida tropicalis AB437087	—	—	—	—	—	—	—
Candida tropicalis	16	1	2	2	1	1	<0.5
Candida tropicalis	16	1	2	2	1	1	<0.5
Candida albicans	16	2	<0.5	1	1	<0.5	<0.5
Candida albicans	16	2	1	1	1	<0.5	<0.5
Candida albicans 90028	16	2	1	<0.5	1	<0.5	<0.5
Candida parapsilosis	16	1	2	<0.5	4	1	2
Candida parapsilosis	16	1	4	1	>32	1	2
Candida parapsilosis 22019	16	1	1	<0.5	1	1	<0.5
Candida parapsilosis	16	1	4	1	1	1	<0.5
Candida parapsilosis	16	1	2	<0.5	>32	1	<0.5
Candida parapsilosis	16	1	2	1	>32	1	<0.5
Candida guilliermondii	8	2	<0.5	2	2	<0.5	16
Candida guilliermondii FJ515181	—	—	—	—	—	—	—
Candida lusitaniae	8	1	1	1	<0.5	1	1
Candida lusitaniae EF136370	—	—	—	—	—	—	—
Candida lusitaniae	16	1	<0.5	1	<0.5	1	1
Candida lusitaniae	16	1	<0.5	1	<0.5	1	1
Candida krusei	8	2	4	>32	>32	1	<0.5
Candida krusei	8	2	4	>32	>32	1	<0.5
Candida krusei	8	2	4	>32	>32	1	<0.5
Candida krusei 6258	8	2	2	4	16	2	<0.5
Cryptococcus neoformans FJ011544	—	—	—	—	—	—	—
Cryptococcus neoformans	16	1	1	>32	16	2	>32
Cryptococcus neoformans	16	1	1	>32	16	2	>32
Candida glabrata 90028	8	2	2	1	2	<0.5	1
Candida glabrata	8	2	2	16	4	4	1
Candida glabrata	8	2	2	16	4	4	1
Ornithonyssus bacoti AM903318	—	—	—	—	—	—	—

Fig. 2 Cladogram tree displaying the yeast isolates tested in this work with the corresponding MIC values of tested compounds. Sq: squalamine, ASD: aminosterol derivative, AMB: amphotericin B, FLC: fluconazole, ITC: itraconazole, CSN: caspofungin.

1–2 mg/l, respectively (Fig. 2). The MIC values of the currently available antifungals tested varied form <0.5 to >32 mg/l depending upon the yeast species and isolate (Fig. 2). High MIC values were found with many isolates, in particular those of *C. guilliermondii*, *C. lusitaniae*, *C. krusei* and *C. neoformans* species (Fig. 2). With respect to toxicity, the half maximal haemolytic concentration (IC_{50}) of squalamine, ASD and AMB were 90, 40 and >100 mg/l, respectively. The mean IC_{50} /MIC ratios were 8 and 30 for squalamine and ASD, respectively. The means $\pm$ standard deviations from triplicate time-kill assays are plotted in Fig. 3. For strains of both *C. albicans* and *C. glabrata*, a fungicidal effect was noted at 4 h with both ASD and AMB and at 8 h with squalamine. The means $\pm$ standard deviation from triplicate intracellular ATP efflux kinetic assays, indicative of yeast cell membrane disruption, of *C. albicans* upon exposure to CTAB, squalamine, ASD, and amphotericin B are plotted in Fig. 4. ATP efflux peaked at 10 min with CTAB and at 40 min with both squalamine and ASD. In contrast, no ATP efflux was detected upon exposure to AMB.

Discussion

This study demonstrated for the first time that aminosterols have remarkable *in vitro* activity against a variety of yeasts implicated in invasive infections. Interestingly, tested aminosterols showed homogenous activities against the fungal species in contrast with the heterogeneous effects of currently used antifungals depending on the fungal species, which agrees with previous reports. [14,15]. This probably indicates that the antifungal effects of aminosterols might enclose new mechanistic aspects that are distinct from those reported with commonly used antifungals. A previous work indicated that aminosterols probably act by disrupting the membranes of Gram-negative bacteria in a detergent-like non-specific manner [5,16]. Thus, their haemolytic effect was regularly used as indicator of their *in vitro* toxicity [16,17]. We found in this work that IC_{50} values for squalamine and ASD were clearly higher than their MIC values suggestive of an acceptable *in vitro* toxicity/activity ratio. However, it should be noted that evaluation of the *in vitro* activity of these compounds according to their haemolytic

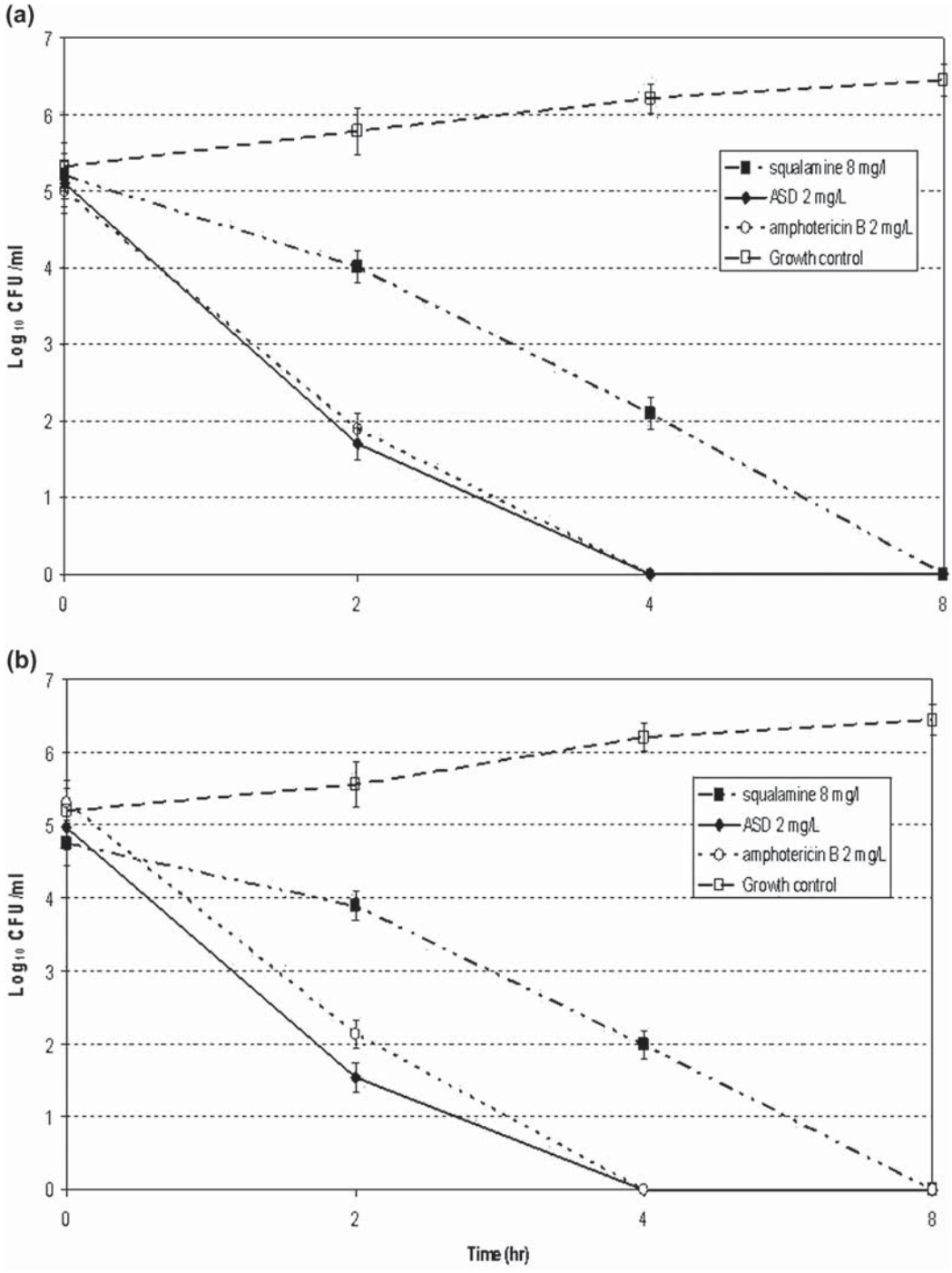


Fig. 3 Time-kill assays of squalamine, aminosterol derivative ASD and amphotericin B tested at their MIC values on *Candida albicans* ATCC 90028 (a) and *C. glabrata* ATCC 90030 (b). Assays were performed in triplicate.

effects remains preliminary and should be validated using more specific tests investigating cellular toxicity, such as colorimetric cytotoxicity assays [18]. Remarkably, fungal killing with ADS occurred as fast as with AMB and faster than with squalamine. Both squalamine and ASD triggered an intracellular ATP efflux indicative of yeast cell membrane disruption as previously demonstrated with bacteria. In contrast, no effect on ATP efflux was noted for AMB [5]. This efflux with squalamine and ASD was much slower and more progressive than that observed with the detergent agent CTAB, indicating that aminosterols may act by disturbing yeast membrane in a detergent-like manner.

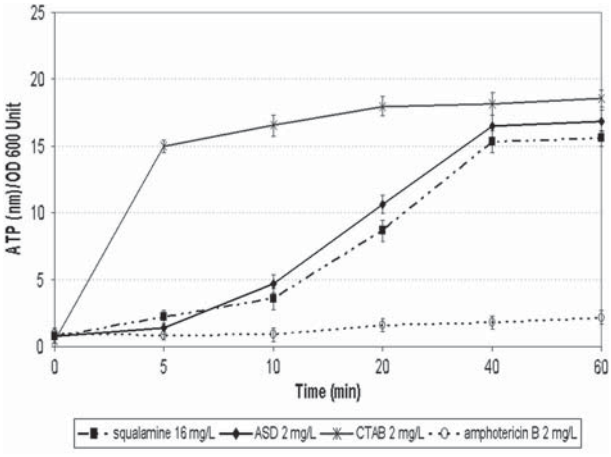


Fig. 4 Kinetics of intracellular ATP efflux from *Candida albicans* ATCC 90028 treated with squalamine, aminosterol derivative ASD, cetyl trimethylammonium bromide CTAB and amphotericin B AMB, all at their MIC values. Assays were performed in triplicate.

This might also explain their haemolytic effect and their activity against strains that were resistant to tested antifungal drugs.

In conclusion, our findings indicate that aminosterol compounds have an interesting antifungal activity probably involving a novel mechanism of action. The cellular toxicity of these compounds should be investigated in the future. Further work is warranted to fully elucidate their mechanism of antifungal effect and to evaluate their *in vivo* activity using experimental fungal infection models.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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Chapitre II : Partie 2

Les diverses applications des dérivés bis(polyaminostéroïdiens) analogues de la squalamine en thérapeutique.

- **Squalamine ointment for *Staphylococcus aureus* skin decolonization in a mouse model**

  Djouhri-Bouktab L, Alhanout K, Andrieu V, Raoult D, Rolain JM, Brunel JM.

Journal of Antimicrobial Chemotherapy. 2011; 66(6):1306-10

- **Squalamine soluble tablets for rapid disinfection of home nebulizer from cystic fibrosis patients**

  Lamia Djouhri-Bouktab, Kamel Alhanout, Véronique Andrieu, Stremler N., Dubus J.C., Raoult Didier, Jean Marc Rolain* and Jean Michel Brunel*

Article 4

Squalamine ointment for *Staphylococcus aureus* skin decolonization in a mouse model.

Lamia Djouhri-Bouktab, Kamel Alhanout, Véronique Andrieu, Didier Raoult, Jean-Marc Rolain, Jean-Michel Brunel.

Journal of Antimicrobial Chemotherapy. 2011; 66(6):1306-10

Résumé de l'article

Le portage nasal constitue un risque majeur d'infection à *S. aureus* qui sévit dans le milieu hospitalier. Le dépistage des porteurs sains ainsi que la décolonisation nasale et cutanée en utilisant des antibiotiques comme la mupirocine et des lavages par des solutions d'antiseptiques est une des stratégies utilisées pour réduire les risques d'infections post chirurgicales. Néanmoins, des résistances à la mupirocine ont été rapportées, d'où la nécessité de la recherche de nouvelles molécules efficaces dans ce domaine.

Dans cette étude, nous avons évalué pour la première fois les activités antibactériennes de la squalamine et d'un dérivé 3,20-bis(polyaminostéroïdiens) *in vivo*. Nous avons ainsi formulé des pommades contenant 1 % de squalamine ou de dérivé bis(polyaminostéroïdien) et développé un modèle animal de colonisation cutanée à *S. aureus* chez la souris. Typiquement les souris sont rasées puis une suspension bactérienne de *S. aureus* calibrée à 10^4-10^6 cfu/mL est déposée sur la peau des souris sur une surface de 9 mm^2. La colonisation à *S. aureus* est stable pendant 2 jours avant d'être naturellement éliminée. Une application unique de pommade de squalamine est capable de réduire de 4 Log les cellules viables de *S. aureus* en 1 heure, alors que la mupirocine n'a permis de réduire dans le même temps que 1.3 Log de cellules viables ($P < 0.05$). La squalamine agissant par un phénomène "mécanique" réduit ainsi la probabilité d'apparition de souches résistantes à cet agent antimicrobien.

Nos résultats suggèrent que cette famille de molécules pourrait être utilisée comme une alternative aux antibiotiques et aux antiseptiques classiques dans la décolonisation cutanée et nasale.

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Squalamine ointment for *Staphylococcus aureus* skin decolonization in a mouse model

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Objectives: *Staphylococcus aureus* colonization of the skin and the nostrils remains a major cause of surgical-site infections despite preoperative and preventive procedures. To date, many compounds have been used for *S. aureus* decolonization, including mupirocin ointments and antiseptics, with variable results. The emergence of mupirocin-resistant *S. aureus* strains has led to the search for new antimicrobial agents specifically for *S. aureus* decolonization. In this work we evaluated squalamine and related parent-derived ointments (1%) as potential new compounds for *S. aureus* decolonization in a new mouse model.

Methods: We report the development and application of squalamine and related parent-derived ointments in a new mouse skin model. After skin shaving, mice were colonized with an *S. aureus* suspension that was calibrated to 10^4–10^6 cfu/mL. The remaining bacterial load was monitored for 2 days after a single application of squalamine by spreading.

Results: We found that *S. aureus* colonization of the skin was stable for at least 2 days before it was naturally eliminated. Using this model we found that squalamine ointment (1%) could reduce *S. aureus* viable cells by up to 4 log with a single, 1 h application of ointment, whereas mupirocin application reduced viable cell numbers by only 1.3 log during that same time ($P < 0.05$).

Conclusions: Our results suggest that such compounds may be useful for *S. aureus* nasal and skin decolonization and may constitute a potent alternative for skin and nasal antisepsis before surgery.

Keywords: aminosterol derivatives, *S. aureus*, mouse skin model

Introduction

Staphylococcus aureus is a human pathogen that colonizes multiple body sites such as the skin and nasal mucous membranes.[1] *S. aureus* colonization constitutes a risk factor for patients undergoing surgery.[2] Indeed, vascular and osteoarticular post-operative infections are often transmitted by asymptomatic chronic carriers with mucosal reservoirs, especially in the nasal mucosa.[3] Thus, preoperative skin and nasal antiseptic strategies remain the most common and imperative medical preventive procedures to reduce post-surgical infections.[4] In past decades, antibiotics such as rifampicin, mupirocin and fusidic acid have been widely used to eradicate *S. aureus* colonization of the nostrils and the skin.[5–9] Nevertheless, antibiotic resistance, especially mupirocin resistance, which was first reported in 1987, has now been reported in many cases.[5,10,11] An alternative to mupirocin is lysostaphin cream; lysostaphin is an antibiotic that cleaves the cross-linking pentaglycine bridges in *Staphylococcus* cell walls. Lysostaphin cream has been shown to be very efficient compared with mupirocin in a mouse model of *S. aureus* nasal decolonization.[12] Other compounds, such as antiseptics, have also been used, including chlorhexidine alcohol and povidone-iodine administered with mupirocin, with rates of up to 75% methicillin-resistant *S. aureus* (MRSA) decolonization in human patients.[13,14] The major advantage for the use of antiseptics is their ability to possess broad-spectrum activity[13] and reduce the risk of surgical infection in hospitals, as well as acting on multiple cellular targets. Their major disadvantage is they are flammable alcohol-based products. Because of the emergence and spread of resistance to these compounds, there is a need to develop new alternative compounds for preoperative preventive measures for *S. aureus* decolonization.

In this context, squalamine (Figure 1), a water-soluble natural polyaminosterol isolated from the tissues of the dogfish shark (*Squalus acanthias*) has shown a wide spectrum of antimicrobial activity, especially against *S. aureus*.[15] The synthesis of squalamine is complicated and yields insufficient output.[16] We were able to demonstrate that numerous synthetic aminosterol

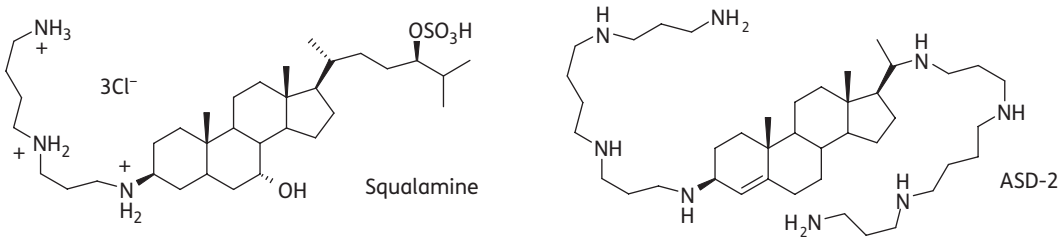

Figure 1. Structures of squalamine and ASD-2.

derivatives, such as aminosterol derivative 2 (ASD-2), possess similar, or better, antistaphylococcal activity against a large panel of *S. aureus* clinical isolates from the sputa of cystic fibrosis patients.[17] Moreover, the dramatic physical effect of squalamine against *S. aureus*, as recently described,[18] may represent an advantage for the development of such compounds for topical use. The aim of our study was to evaluate and compare the well-known antibiotic squalamine and an aminosterol derivative, which were both added individually to ointments, as potential new drugs for rapid *S. aureus* decolonization in a new mouse skin model.

Materials and methods

Compounds

Squalamine was a gift from Professor M. Zasloff (Georgetown University, Washington, DC). ASD-2 was synthesized according to the previously reported procedure[19] (Figure 1). Stock solutions (2 g/L) of squalamine and ASD-2 were prepared in water and methanol, respectively. Stock solutions were subsequently diluted in water to 250 mg/L working solutions. The antibiotic controls used in this study were vancomycin (Merck Génériques, Lyon, France), mupirocin ointment (Bactroban 2%; GlaxoSmithKline Laboratory, France) and fusidic acid ointment (Fucidine 2%; Leo Laboratory, France).

Bacterial strains

Reference bacterial strains used were methicillin-susceptible *S. aureus* (ATCC 25923) and MRSA CF Marseille (CSUR P102).[20] Susceptibility testing MICs were determined in duplicate by the broth microdilution method, conducted according to BSAC guidelines, providing a limit of detection of 60 cfu/mL.[21] Bacteria were cultivated on Trypticase soy agar (TSA) plates for 24 h at 37°C.

In vitro selection of aminosterol-resistant S. aureus

Squalamine and ASD-2 were successively diluted in 5 mL of Mueller–Hinton medium to different concentrations ranging from 0.5–4 times the MIC. Suspensions of susceptible *S. aureus* bacterial strains (ATCC 25923 and CF Marseille CSUR P102) were added to each tube. Bacterial growth in the presence of aminosterol derivatives was verified by obtaining a culture from 10 μL samples on TSA plates.

Selection of mupirocin-resistant S. aureus

Selection of a mupirocin-resistant strain of *S. aureus* was performed using the CF Marseille *S. aureus* strain (CF Marseille CSUR P102), which was available in our laboratory. Susceptibility to mupirocin was estimated by culturing the bacteria in the presence of mupirocin discs (5 μg); the diameters of the zones of growth inhibition were measured after incubation at 37°C for 24 h.

After 24 h, colonies that had grown around the disc were resuspended in 3 mL of Luria–Bertani broth and incubated for 3 h at 37°C. The suspension was calibrated to 10^6 cfu/mL and spread on TSA plates, and a mupirocin disc was deposited in the centre of the plate. This procedure was repeated until complete resistance to mupirocin developed, i.e. observation of growth in contact with the mupirocin discs.

Preparation of aminosterol and vancomycin ointments

The aminosterol and vancomycin ointments (1%) were prepared by mixing 9.9 g of petrolatum-based cream (Cooper, Cooperation Pharmaceutique Française, France) and 100 mg of the desired active substance. Briefly, 100 mg of squalamine or the aminosterol derivative were weighed and crushed in a mortar. Petrolatum-based cream was added gradually and the contents were mixed for 10 min until a homogeneous ointment was obtained. Finally, the ointment was stored in a sterile tube at 4°C. Absence of bacterial contamination in the different ointments was verified by suspending them in sterile distilled water, depositing 100 μL of each suspension on a TSA plate and incubating the plate at 37°C for 24 h. Absence of any bacterial growth after 48 h of culture incubation was considered a marker of ointment sterility.

Mouse model of S. aureus skin colonization

Cutaneous colonization was studied exclusively in 4–8-week-old female BALB/c mice; inbred strain (Charles River Laboratory, France). Each assay with a tested compound was carried out with five mice in two independent experiments. Mice were anaesthetized with ketamine (25 mg/kg) and xylazine (3 mg/kg) (Bayer and Panpharma Laboratory, respectively), and a 9 cm^2 area on the back dorsal cervical surface was shaved with an electric razor. The shaved area was divided into two equal zones.

In the first set of experiments, 10 μL of a bacterial suspension of *S. aureus* calibrated to 10^8 cfu/mL was applied to the skin of the shaved mice on the two different zones. After 1 h, 20 mg of pure petrolatum-based cream (control) or the aminosterol-derivative ointment was spread in a single application on the left and right zones, respectively. For some experiments, antibiotic ointments were used in place of the aminosterol-derivative ointments. Colonization was monitored by swabbing the skin of the mice once daily with a dry swab (sterile swab, wooden applicator and cotton tip; Copan Italia S.p.A., Brescia, Italy) after 0, 24, 48 and 72 h. The swabs were inoculated in 1 mL of physiological serum, 10-fold serial dilutions were performed and 100 μL of each dilution was plated on Chapman agar plates. These plates were incubated at 37°C for 24 h before bacterial enumeration (cfu/mL).

In a second set of experiments, 10 μL of a *S. aureus* bacterial suspension calibrated to 10^6 cfu/mL was applied to the skin of shaved mice, as described above. After 1 h, 20 mg of pure petrolatum-based cream (control) or cream containing an antibiotic (vancomycin, fusidic acid or mupirocin) or the aminosterol derivative ASD-2 was spread in a single

Table 1. MIC values of squalamine, ASD-2 and classical antibiotics for *S. aureus* strains

Strains	MIC (mg/L)			
	squalamine	ASD-2	mupirocin	vancomycin
S. aureus ATCC 25923	2	2	0.5	1
S. aureus CF Marseille CSUR P102	2	2	0.5	1
S. aureus CF Marseille CSUR P102, mupirocin-resistant	2	2	100	0.5

application, as previously described. Colonization was monitored after 0, 30 and 60 min, and bacteria were counted as described above.

This study (registry number: 2-28012011) was approved by our local Ethics Committee (Faculty of Medicine, Université de la Méditerranée, Marseille, France).

Results

As shown in Table 1, squalamine and ASD-2 possess similar activity against susceptible and methicillin-resistant CF Marseille *S. aureus* strains, with 2 mg/L MIC values in all cases. We were not able to select for an *in vitro* aminosterol-resistant *S. aureus* strain even after having repeated the experiment more than 20 times. Conversely, a mupirocin-resistant *S. aureus* isolate was obtained by *in vitro* selection on plates with a mupirocin disc after 33 subcultures. The initial mupirocin MIC, as determined using the microdilution method, was 0.5 mg/L, whereas the fully resistant isolate had an MIC value of 100 mg/L. Interestingly, the MIC values of squalamine and ASD-2 were the same for this mupirocin-resistant strain. Squalamine, ASD-2 and vancomycin ointments (1%) were prepared extemporaneously for each experiment, and the sterility of each cream was verified by microbiological analyses that demonstrated no bacterial growth.

For the mouse skin model, brief anaesthesia was necessary to shave the mice properly and to apply the bacterial suspension. In the first set of experiments we applied 10 μL of a bacterial suspension (10^6 cfu/mL) to the skin to monitor the natural elimination of bacteria over 3 days. Bacterial enumerations during this challenge showed that the bacterial load remained stable for at least 2 days (10^6 cfu/mL) and then naturally decreased to 10^2 cfu/mL by day 3 (Figure 2a). Thus we studied the efficacy of squalamine and aminosterol ASD-2 ointment on *S. aureus* colonization over the course of 3 days. A single application of squalamine and ASD-2 ointment was able to reduce *S. aureus* viable cells by up to 4 log compared with the control ($P<0.05$) 2 days after application. In the second set of experiments we studied and compared the rapidity of action of squalamine and ASD-2 with various antibiotics for skin decolonization after 30 and 60 min (Figure 2b). Interestingly, a single squalamine ointment application was able to reduce the bacterial load of *S. aureus* viable cells by 4 log after only 30 min. Fusidic acid, mupirocin and ASD-2 reduced the bacterial population by 1.7, 1.3 and 1 log after 1 h, respectively, under the same experimental

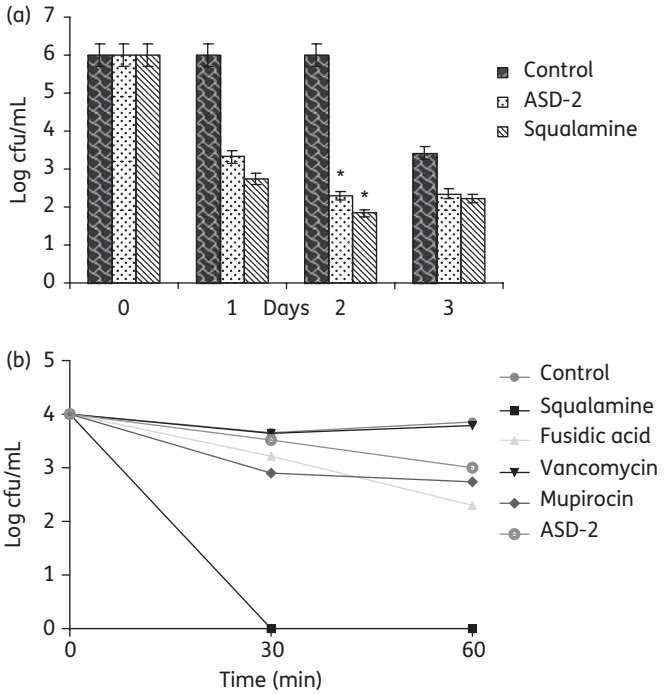

Figure 2. *S. aureus* bacterial enumeration after application of ointments containing squalamine or an aminosterol derivative (ASD-2) over 3 days (a) or over 1 h as compared with fusidic acid, vancomycin and mupirocin (b). Results are means (±SD). Asterisks indicate significant differences compared with the control.

conditions (Figure 2b). Using a vancomycin ointment (1%), no reduction in bacterial growth was observed after 1 h. This result was similar to that obtained with the control. Finally, no significant differences were noticed in terms of the appearance of treated or untreated skin patches, and no lesions or inflammation were encountered after the application of aminosterol-derivative ointments.

Discussion

Testing antistaphylococcal therapeutics for skin and/or nasal decolonization generally requires a suitable animal model. Several animal models for nasal colonization have been previously reported, but such models are usually time consuming and labour intensive. Thus these models are not suitable for the rapid screening of new compounds. Moreover, it has been demonstrated that mouse nasal colonization models are not easy to implement, and high levels of colonization could not be achieved using such models. These models have also been shown to be inconsistent and not reproducible.[22,23] The cotton rat model has been used as an alternative model for nasal colonization and has proven to be reproducible with higher levels of persistent *S. aureus* nasal colonization, i.e. bacterial loads ranging from 10^3–10^4 cfu/nose that may persist up to 6 weeks.[24] However, this model is very fastidious, since it requires intranasal *S. aureus* instillation, animal treatment, nostril bisection with scissors and an *S. aureus* culture[24] that is not suitable for rapid screening. This is the reason why we investigated the

development of an easier and faster mouse skin model to screen our compounds. We found that our model was easy to implement by just shaving mice and applying bacterial suspensions and ointments directly to the skin. This model is efficient, as it yields a reproducible level of *S. aureus* colonization, ranging from 10^4–10^6 cfu/mL over the course of 2 days, comparable to levels in the cotton rat model. Natural elimination of bacteria occurred after 3 days in our model. It is well known that staphylococcal skin infections often clear spontaneously, with a peak in *S. aureus* charge at 2–4 days and a rapid decline thereafter.[22] For this reason, our mouse skin model cannot be used as a chronic *S. aureus* colonization model, but rather as a rapid test to screen potential active compounds.

Squalamine has reached Phase III trials for the treatment of age-related macular degeneration and prostate cancer disease without any major side effects, as it appears to be well tolerated even at doses 250 mg/day in adults, suggesting that an ointment for local use could be totally safe. Comparisons between the petrolatum-based cream (control), squalamine and ASD-2 ointment revealed a high degree of bacterial load reduction (up to 4 log, bactericidal effect) of viable *S. aureus* cells during a 2 day experiment. These results suggest the possible use of these ointments for *S. aureus* decolonization. Moreover, we found that squalamine rapidly killed bacteria, with a single application of ointment reducing bacterial load by up to 4 log after only 30 min. Fusidic acid is known to have little or no bactericidal effect on *S. aureus in vitro*,[25] whereas a 2 log reduction for mupirocin and a 3 log reduction for vancomycin have been reported after 24 h in a time–kill study assay.[26] In the study by LaPlante,[26] bacterial load reductions at 4 h for mupirocin and fusidic acid were less than 1 log. The rapid bactericidal effect of squalamine could be attributable to the dramatic depolarizing effect observed on *S. aureus*, which resulted in rapid cell death.[18] Although ASD-2 possesses the same *in vitro* activity against *S. aureus* as squalamine, ASD-2 does not decolonize *S. aureus*-colonized skin as quickly as squalamine. In terms of time efficiency, this difference may be due to the structure of the aminosterol derivative or, more probably, its diffusion ability (ASD-2 is soluble in methanol). However, many other aminosterol derivatives, including water-soluble compounds, have already been synthesized and may be screened in the future using our model to select a more potent compound. Lysostaphin has also been used in a cotton rat model for *S. aureus* colonization. A single application of this glycylglycine endopeptidase at 0.5% in petrolatum-based cream was able to eradicate *S. aureus* colonization in 93% of animals after 4 h.[12] Finally, a novel chimeric bacteriophage lysine used in a mouse nasal decolonization model was able to reduce viable *S. aureus* cells by up to 2 log 1 h after a single application.[27] Thus, squalamine ointment appears to be a potent alternative to mupirocin for the eradication of *S. aureus* on the skin and in the nose. A single dose of squalamine (formulated at 1% in a petrolatum-based cream) eradicates *S. aureus*, whereas multiple applications of mupirocin over 3–5 days are usually required to obtain a significant reduction in nasal colonization. This fact precludes the use of mupirocin for patients requiring emergency surgical procedures. The rapidity of action of squalamine may be advantageous in these situations. Moreover, squalamine and its related derivative ASD-2 had the same *in vitro* activity against our local mupirocin-resistant isolate. This suggests that decolonization may also be effective in cases of mupirocin-resistant strains. At the same time, all of our attempts to select aminosterol-resistant *S. aureus* strains failed. This is probably because of the original physical mode of action of the aminosterol derivatives, which target the integrity of the bacterial membrane. This particular mode of action severely limits the possibility that the bacteria could bypass the drug's mechanism.[18]

Conclusions

In summary, our mouse skin colonization model may be a valuable tool for screening compounds that may have potent activity for *S. aureus* skin decolonization. With this model we have demonstrated that squalamine in a cream base may be an alternative to mupirocin ointment for the eradication of *S. aureus* skin colonization. Finally, our results support the development of such creams for the rapid eradication of *S. aureus* skin and nasal colonization for the prevention of nosocomial *S. aureus* infections. Nevertheless, even if these data are promising, they still remain preliminary in nature.

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Transparency declarations

None to declare.

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Article 5

Squalamine soluble tablets for rapid disinfection of home nebulizer from cystic fibrosis patients

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Résumé de l'article

La mucoviscidose est une maladie génétique caractérisée par des infections récurrentes des poumons, le traitement de cette maladie nécessite des thérapies antibiotiques par voie aérosol au moyen de nébuliseurs. Cependant, des germes pathogènes provenant du tube digestif du patient sont fréquemment retrouvés dans le matériel de nébulisation et cette contamination microbienne serait à l'origine de la réinfection du patient et de l'échec des traitements. Ainsi à l'heure actuelle, le nettoyage et la désinfection du matériel de nébulisation sont recommandés. La désinfection peut être réalisée par immersion dans des solutions contenant de l'hypochlorite de sodium (0.15%), de l'acide acétique (2%) ou dans de l'eau bouillante.

Dans ce travail, nous avons évalué pour la première fois l'utilisation de la squalamine en tant que désinfectant des nébuliseurs infectés par une suspension bactérienne (*S. aureus* and *P. aeruginosa*) calibrée à 10^8 cfu/mL ou fongique (*C. albicans* and *A. niger*) calibrée à 10^7 cfu/mL. La désinfection est réalisée par immersion du nébuliseur dans une solution de squalamine pendant 20 min. Le glutaraldehyde et le korsolex (acide peracétique) servant de contrôle d'inhibition, la squalamine utilisée à une concentration de 0.5 g/L est capable de réduire de 5 Log_{10} les cellules viables de bactéries telles que *S. aureus, P. aeruginosa* et de 4 Log_{10} les champignons tel que *C. albicans* pour un cycle de 20 min. En revanche, dans le cas d'*A. niger* la concentration permettant d'obtenir un effet fongicide est supérieure à celle des bactéries avec un temps plus lent (2 g/L durant 6 heures).

Des cachets hydrosolubles de squalamine (2.5 %) ont été formulés avec des résultats comparables pour faciliter la méthode de désinfection du matériel de nébulisation.

En conclusion, la squalamine apparaît comme une solution potentielle simple, rapide et efficace pour la désinfection de tout matériel susceptible d'être contaminé. Une extension possible de ce travail serait d'évaluer directement l'activité bactéricide et fongicide de la squalamine sur des nébuliseurs infectés provenant de patients mucoviscidosiques afin d'évaluer les activités bactéricides et fongicides des analogues de la squalamine.

Abstract

Background: Bacterial contamination of nebulizers represents a major problem for cystic fibrosis patients leading to reduced nebulizer performance and increasing the risk of patient reinfection by the contaminant bacteria.

Objective: We investigated herein the potential use of broad spectrum antimicrobial squalamine compound *in vitro* in a nebulizer disinfection model.

Methods: Pari LC nebulizers were infected by bacterial (*S. aureus* and *P. aeruginosa*) calibrated at 10^8 cfu/mL and fungal (*C. albicans* and *A. niger*) suspension calibrated at 10^7 cfu/mL and disinfected by immersion in squalamine solution during 20 min. Glutaraldehyde and korsolex peracetic acid were used as inhibition control.

Result: We found that squalamine 0.5 g/L was able to reduce 5 Log_{10} of *S. aureus, P. aeruginosa* and 4 Log_{10} *C. albicans* viable cells in 20 min while we need a concentration at 2 g/L for reduce 4 Log_{10} of *A. niger* cells in 6 hours. Finally, a formulation of squalamine disinfecting soluble tablets at 2.5 % (W/W) was developed and successfully applied for nebulizer disinfection.

Conclusion: Our results suggest that this family of compounds may be used by cystic fibrosis patients for rapid and easy home nebulizer disinfection and that soluble tablets may be developed for this purpose.

Keywords: nebulizer, cystic fibrosis (CF), aminosterol derivatives, disinfection

Introduction

Cystic fibrosis (CF) is characterized by recurrent lung infections requiring repeated inhaled therapies using devices, namely nebulizers, transforming drug liquid formulations into an aerosol form inhaled by the patient [1-3]. Several compounds were used for aerosol therapy such as antibiotics, bronchodilators and mucolytics [4]. Since nebulizers are frequently used by the patients for considerably long periods, microbial contamination generally occured. It is a serious problem, leading to reduced nebulizer performance and increasing the risk for patient reinfection [1, 5-7]. Thus, to prevent microbial contamination of nebulizers, such devices should be disinfected [8] and few guidelines for nebulizer disinfection are available [8], some recommending a soaking in vinegar solution, boiling water [9, 10] or hypochlorite solution [11, 12]. Such procedures are not easy and not convenient for patients that should disinfect their nebulizer every day. Simple procedures may be developed to facilitate the routine practice of nebulizer disinfection to avoid complicated cleaning procedures hardly followed by patients and/or their families. In this context, squalamine has been recently described to possess broad spectrum antimicrobial properties against various multidrug resistant bacterial and fungal isolates recovered from CF patients [13, 14]. It has been recently reported that squalamine acts as a membrane-active molecule targeting bacterial membrane integrity[15] . Because of its original mode of action, acting as a detergent, its antimicrobial activity appears very large against multi drug resistant bacteria and fungi usually recovered from sputum

samples of CF patients [15]. Broad spectrum antimicrobial activity of squalamine suggests that this compound may be a good candidate for its development as disinfecting agent. In the present study, we evaluated for the first time the suitability of squalamine on aqueous solution for commercial home nebulizer disinfection, using *P. aeruginosa*, *S. aureus*, *C. albicans* and *A. niger* as reference strains and protocol recommended by European standards. Finally, water soluble squalamine tablets were developed and tested in this simple and rapid cleaning and disinfecting model.

Material and Methods

Microbial strains and general procedures

Antimicrobial activity of squalamine compound was determined in duplicate by broth microdilution method as previously described [16]. *Pseudomonas aeruginosa* DSM 939 (ATCC 15442), *Staphylococcus aureus* DSM 799 (ATCC 6538), *Candida albicans* DSM 1386 (ATCC 10231), and *Aspergillus niger* ATCC 16404 were used as reference strains following guidelines of the French and European standards (NF EN 1040 and NF EN 1275, 1997). Bacteria and fungi were cultivated on tryptone soy agar and Sabouraud agar during 24 hours and 48 h, respectively. Bactericidal properties were defined when a reduction of 5 $\log_{10}$ was obtained for *S. aureus* (ATCC 6538) and *P. aeruginosa* (ATCC 15442) strains from a calibrated solution of 10^8 cfu/mL during a defined time. On the other hand, fungicidal properties were defined when a reduction of 4 $\log_{10}$ was obtained for

C. albicans (ATCC 10231) and *A. niger* ATCC 16404 from a calibrated solution of 10^7 cfu/mL by using a dilution-neutralization or a membrane filtration method (NF EN 1040 and NF EN 1275, 1997).

Drug compounds

Squalamine was obtained from a generous gift from Pr. M. Zasloff (Georgetown University, Washington). Stock solutions were prepared in water for squalamine. Stock solution was subsequently diluted to 250 mg/L working solutions. The control of inhibition efficiency was performed using korsolex peracetic acid (Bode Chemie GmbH, Hambourd, Germany) and glutaraldehyde (2%) (Electron Microscopy Sciences, Hatfield, USA) as controls as recommended by CCLIN 2001 for endoscopes disinfection.

Protocol of disinfection

Microbial contamination of nebulizer was involved *in vitro* by immerse the inner part of nebulizer (Pari LC, SPRINT SP, Pari, Germany) in a bacterial suspension prepared in Mueller Hinton broth containing 1.10^8 to $5.\ 10^8$ cfu/mL of bacteria (NF EN 1040, 1997) (figure 1). Then, disinfection was realized by soaking the same part in sterile water containing squalamine at determined concentrations depending on the considered strains. For fungi 1.10^7 to $5.\ 10^7$ cfu/mL suspensions (NF EN 1275, 1997) were prepared in Sabouraud broth and after incubation the nebulizer was immersed in sterile water containing squalamine. The nebulizer was then soak in two successive

sterile water solutions and removed. Subsequent bacterial or fungal enumeration of the second immersion water bath was then realized. Enumeration of bacterial or fungal colonies was done on Tryptone Soy Agar for bacteria growth and malt extract agar for fungi. The bactericidal and fungicidal test was performed three times in independent experiments and each bacteria and fungi were tested separately. The concentration of squalamine was increased until obtaining bactericidal and fungicidal activity. The time of disinfection was defined in the first to 20 min, and then extended to 6 hours for *A. niger*.

Squalamine tablets manufacture

A batch size of 20 g was prepared; the mixture was realized in a mixer Turbula Type T 20, Switzerland. The tablets were manufactured in an alternative tabletting press Korsch Erwika Type EKO, Germany. In the first the raw materials was sieved. Squalamine (0.5 g) was mixed with microcrystalline cellulose (7.2 g), lactose (10 g), starch (1.4 g), sodium croscarmellose (0.6 g) and colloidal silica (0.04 g). Then, sieved magnesium stearate was added and mixed. The final blend is compressed on an alternative tablet press equipped with 8.0 mm punches. Excipients with specific particle size dedicated to direct compression were used. Tablets were kept in polyethylene low density bottle.

Disinfection experiments were done less than 10 days after squalamine tablets manufacture.

Results

In a preliminary set of experiments, MIC values for squalamine were determined against the selected reference bacteria and fungi strains. The MIC values were 2, 4, 16 and 16 mg/L against *S. aureus*, *P. aeruginosa*, *C. albicans*, and *A. niger*, respectively (Table 1).

The bactericidal and fungicidal activity was performed by infecting nebulizer with a bacterial suspension (1.10^8 to $5.\ 10^8$ cfu/mL) or a fungal suspension (1.10^7 to $5.\ 10^7$ cfu/mL) and disinfected by a subsequent immersion in a squalamine solution. We defined 20 min for time nebulizer disinfection by using squalamine whereas korsolex PAA and glutaraldehyde (2%) solution were used during 15 min to obtain disinfectants properties.

In the first attempt, we determined that a 80 mg/mL dose for 20 min led to a reduction of 2 and 3 Log for bacteria whereas only a reduction of 2 log for *C. albicans* were obtained using 190 mg/mL squalamine concentration. In the same time, no significant decrease of *A. niger* viable cells were noted under these conditions. The reduction of bacteria and fungi obtained with this concentration of squalamine was lower than those recommended by the standards (5 Log for bacteria and 4 Log for fungi). Thus, we have increased squalamine concentration until obtaining bactericidal and fungicidal activity; a five Log_{10} reduction for *S. aureus* and *P. aeruginosa* was obtained using 210 and 130 mg/L concentration of squalamine for 20 min treatment whereas concentration of 500 mg/L and 2 g/L was necessary

in order to obtain a reduction of 4 Log_{10} for *C. albicans* and *A. niger* viable cells after 20 min and 6 hours, respectively (figure 1).

In the case of *A. niger*, the time was firstly fixed to 20 min and the dose was increased until 1 g/L but no fungicidal activity was observed under these conditions. In a second set of experiment, we have increased the time to 6 hours and the dose of squalamine used for nebulizer disinfection, from 1 mg/mL to 2 mg/mL leading to a reduction of 3 and 4 Log for this fungal strain, respectively. Furthermore, it is noteworthy that no growth was observed by using korsolex peracetic acid and glutaraldehyde 2 %.

On the other hand, white to almost white flat round soluble squalamine tablets weighing 200 mg with a hardeness of 8.7 Kp, containing 2.5 % of squalamine with a disintegration time of 5 min in water at ambient temperature have been successfully formulated. Thus, three squalamine tablets dissolved in 50 mL sterile distilled water were able to reduce of 5 Log_{10} of viable bacteria cells whereas six squalamine tablets reduce 4 log_{10} of *C. albicans* in 20 min.

Discussion

Nebulizers are widely used by CF patients and are well considered as a source of contamination in cystic fibrosis [17]. Cleaning and disinfection have been recommended to limit the risk of contamination [7]. Various recommendations were proposed by different foundations and have been used for nebulizer disinfection including steeping daily in vinegar solution (2%), boiling in water [9, 10]

and washing machine at 70°C [11]. Other methods include immersion in 70 % to 90 % ethyl or isopropyl alcohol for 5 min [8] and soaking and rinsing with tap water followed by air drying [18, 19]. Sodium hypochlorite was also recommended but with different standards depending on the country. Thus, the French cystic fibrosis association recommends the use of hypochlorite solution at 0.08 % of active chlorine during 15 to 30 min whereas the American cystic fibrosis foundation, recommends the immersion of the infected material in a solution at 0.13 % of active chlorine during 3 min [11]. In 2001, Rosenfield et *al.* were the first to study the contamination of jet nebulizer with cocktail of *S. aureus* and *P. aeruginosa* and to clean them with tap water at 35°C steared for 30 s and dried at room temperature leading to a bacterial density reduction to 20 cfu/mL when the average of positive control was 10^5 cfu/mL [18]. In 2006, a study on patient ultrasonic nebulizers cleaning indicates a lower number of contaminated samples performing the nebulizer disinfection with sodium hypochlorite (100 ppm) during 1 hour once daily in comparison with those disinfected once at intervals of 2-7 days and leading to the conclusion that disinfection of nebulizers after 24 hours was desirable [20]. More recently, Reychler et *al.* have compared the *in vitro* efficiency of different disinfectants against 16 Gram positive and Gram negative bacteria by using hypochlorite solution (0.02% chlorine), acetic acid 3.5 %, Hexanios 0.5%, washing-up detergent (0.5%) during 20 min and dishwasher (70°C) . Disappearance of germs was observed whatever the disinfectant used

(reduction of 5 log) against *S. aureus, P. aeruginosa, Stenotrophomonas maltophilia, Achromobacter xylosoxydans* and *Burkholderia cenopacia*[11].

The initial concentrations of germs in culture broths used for contamination was 7.3 to 8.2 log cfu/mL except for *Burkholderia cenopacia* which was calibrated at 5.2 log cfu/mL. Nevertheless acetic acid (3.5 %) had pronounced odor and was ineffective in disinfecting *S. aureus* and *B. cenopacia* contaminated materials [11].

In the same purpose, Monforte et *al.* demonstrated that a correct disinfection could consist to wash the nebulizer in soap and water after use and immerse it in a sodium hypochlorite (1%) solution until next administration, since only 12.5 % of the nebulizer of patients which have followed this protocol were contaminated whereas 60 % were contaminated otherwise [7].

On the other hand, water sources used for nebulizer rinsing are variable (distilled water, tap water, sterile water) but can be a source of nebulizer contamination since for example *Stenotrophomonas maltophilia* was found in tap water [21] .

In this work, we demonstrated for the first time the suitability of squalamine as disinfecting compound using a nebulizer model. In this context, a reduction of 5 Log of bacterial viable cells and 4 Log of fungal viable cells are needed in order to demonstrate a disinfecting activity. In our study, reduction of fungal growth of *A. niger* requires longer time (6 hours) than for reduction of bacterial growth, in accordance with MICs value of squalamine which were higher in the

case of fungi in comparison to bacteria [22]. *A. niger* is a filamentous fungi which is able to form spore and is reported to be more resistant than other fungi towards glutaraldehyde since 0.5 % of alkaline glutaraldehyde was able to reduce 4 log of *A. niger* spore after only 100 min [23]. Vizcaino et *al.* demonstrated that glutaraldehyde fixed to the matter to the scalpel was able to produce corrosion within 2 hours[24]. No fungal reduction of *A. niger* spore was observed after an exposure time of 4 hours to titanium dioxide layer probably due to the structure of spores containing chitin and glycoproteins [25].

Generally, CF children are very dependent from their parents particularly concerning the nebulizer disinfection task. Since patients used daily nebulizers, disinfection methodology must be simple and practical and should be improved for better compliance [26]. Herein, we have developed a simple, rapid and easy method for home nebulizer disinfection by using squalamine tablets. This could be comparable to disinfecting tablets for daily decontaminations of feeding bottles. Squalamine soluble tablets were successfully used for the decontamination of an infected nebulizer reducing of 5 log the bacterial viable cells after 20 minutes of immersion similarly to the obtained results under homogeneous experimental conditions. It is noteworthy that the formulated tablets also were disintegrated in water solution in less than 5 minutes suggesting their potent use as an easily handled disinfecting agent. Another improvement of this model could be realized by preparing effervescent tablets that may ensure a faster and homogeneous compound distribution in water. A limitation of our

study is due to the use of a new nebulizer whereas used nebulizers from CF patients could be more difficult to disinfect due to their irregular surface.

On an economical point of view, a nebulizer costs around 20 € and this latter must be changed after four months of use in order to maintain correct nebulisation parameters. Under these considerations, the annual cost for nebulizers is estimated to be around 60 €. On the other hand, the cost for 10 squalamine tablets is of 4 € suggesting that the use of such tablets may be cheap. Thus, it clearly appears that squalamine tablets easy and daily used can prevent reinfection of patients by contaminated devices. Tablets squalamine use was ecologic, this aminosterol was present naturally in the shark. This disinfection method may have a psychological impact because patient become independent in nebulizer disinfection and use daily a clean nebulizer.

Conclusion

In summary, our method was easy to involve since it required a simple dumping of contaminated nebulizer directly in a water solution containing squalamine soluble tablets and appears to be effective for nebulizer disinfection in our simple laboratory conditions. It would be interesting to test activity of squalamine tablets directly on contaminated nebulizers used by CF patients and screening bactericidal and fungicidal properties of other aminosterol derivatives for their potential use in nebulizer disinfection.

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Table 1. Antimicrobial activity, bactericidal and fungicidal dose of squalamine.

Strains	MICs (mg/L) of squalamine	Squalamine dose (mg/L) for nebulizer disinfection
S. aureus DSM 799	2	210
P. aeruginosa DSM 939	4	130
C. albicans DSM 1386	16	500
A. niger ATCC 16404	16	2000

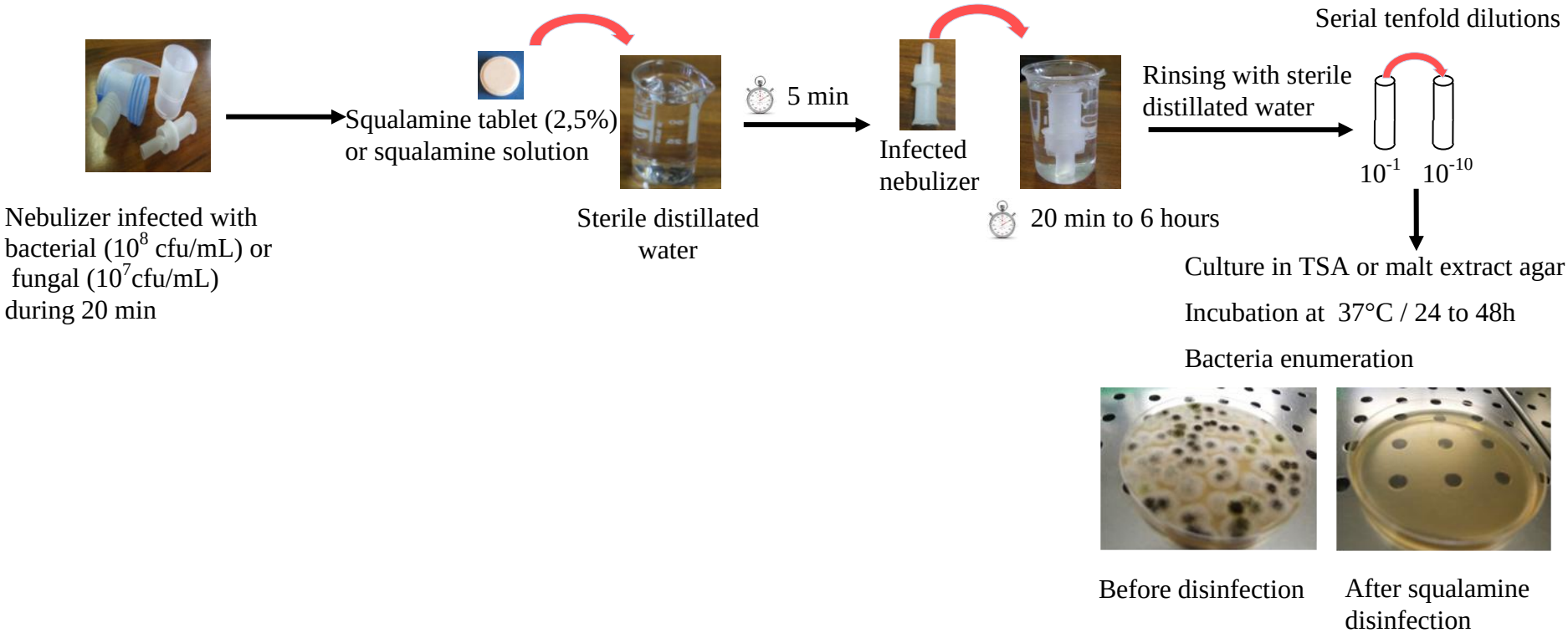

Figure 1. infection and disinfection model of nebulizer

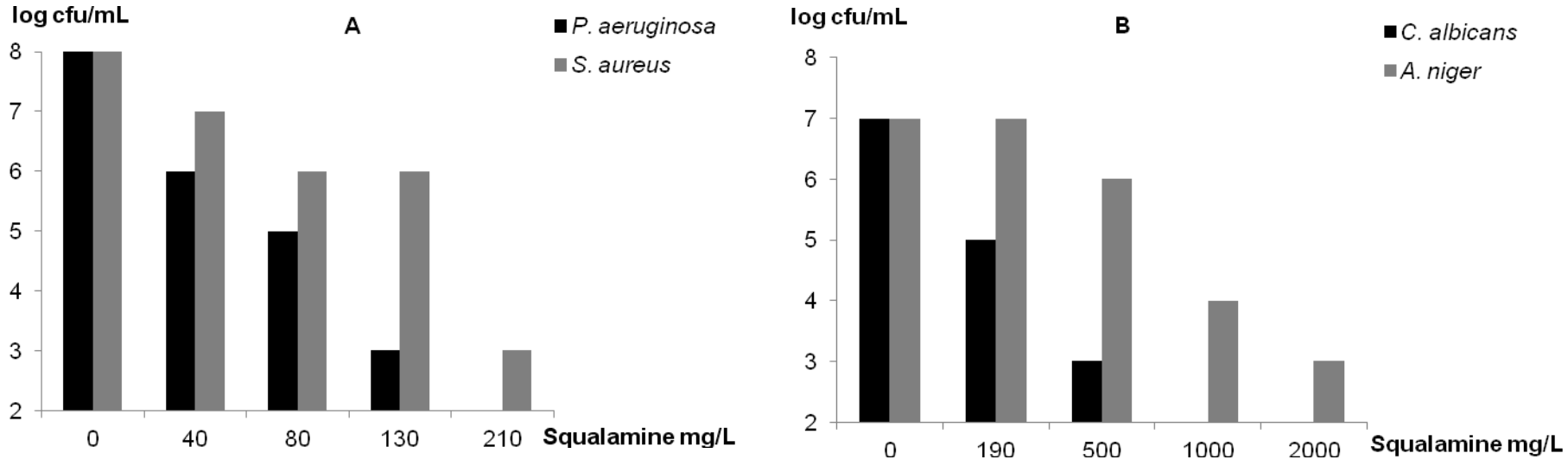

Figure 2: Result of nebulizer disinfection with squalamine solution on bacteria (A) and fungi (B).

Conclusions générales et perspectives

Nous avons réalisé, dans le cadre de ce travail, la synthèse de nouveaux analogues de la squalamine, les dérivés 3,20-bis(polyaminostéroïdiens) et l'évaluation de leurs activités antimicrobiennes vis à vis de nombreuses souches sensibles. Ces composés sont ainsi très actifs vis-à-vis des bactéries Gram positive et Gram négative même si on remarque que les CMIs sont plus élevées dans le cas des souches résistantes à la colistine tel qu'*Iquilinus limosus*. L'étude des relations structure-activité démontre une corrélation entre la longueur de la chaîne polyaminée et l'activité antibactérienne de ces dérivés soulignant par le même mécanisme d'action original ciblant la membrane bactérienne (Article 2). Il serait aussi intéressant d'étendre cette étude aux activités antifongiques de cette famille de molécules. Nous avons également démontré une bonne activité de la squalamine et d'un aminostérol DAS-1 sur divers souches de levures y compris les souches multirésistantes avec un mécanisme d'action qui reste à l'heure actuelle non élucidé sur les champignons même s'il semble probable que la membrane des levures soit la cible de cette famille de molécules.

Les activités antimicrobiennes encourageantes obtenues pour les analogues de la squalamine nous ont poussé à envisager leur

utilisation par voie locale ou dans la désinfection du matériel médical. La squalamine et ces analogues présentent d'excellentes activités antistaphylococciques *in vitro* sur des souches sensibles et résistantes à la méthicilline. L'étude du mécanisme d'action montre que la squalamine et les dérivés aminosteroïdiens induisent une dépolarisation de la membrane des bactéries Gram positive suivi d'un drainage du contenu intracellulaire réduisant ainsi la probabilité de développer des résistants à la squalamine[11]. Dans ce contexte, nous avons développé dans notre laboratoire, un modèle animal de colonisation cutanée à *S. aureus* afin d'évaluer les performances de la squalamine et d'un dérivé bis(polyaminostéroïdien) DAS-2 formulés sous forme de pommade. Ainsi, une seule application de la pommade à base de squalamine réduit significativement la colonisation à *S. aureus* comparé aux antibiotiques classiques tels que la mupirocine et la fucidine. La squalamine et les dérivés bis(polyaminostéroïdiens) pourraient ainsi être développés comme des antiseptiques capables de prévenir et réduire les risques d'infections chirurgicales hospitalières.

Parallèlement, la squalamine et ses analogues ont été utilisés dans le cadre de la décontamination du matériel médical. La squalamine s'est ainsi montré très efficace dans la désinfection de nébuliseurs infectés par diverses souches de *S. aureus, P. aeruginosa* et *C. albicans*. En revanche, dans le cas

d'*Aspergillus niger*, la squalamine s'est montré moins efficace probablement due à la présence de spores contenant de la chitine et des glycoprotéines confèrant une résistance au traitement de désinfection. Il serait intéressant de tester les propriétés bactéricides et fongicides des cachets de squalamine sur des nébuliseurs contaminés provenant des patients mucoviscidosiques afin d'envisager une désinfection systématique et rapide de leurs nébuliseurs. Il serait également intéressant de déterminer les activités de ces dérivés sur les virus, les mycobactéries et les spores.

En conclusion, une perspective ou un travail serait d'évaluer les activités antifongiques des dérivés bis(polyaminostéroïdiens) afin d'envisager leur potentiel utilisation dans le traitement des infections cutanées d'origine fongique et de déterminer également leur mécanisme d'action sur les champignons. Des études récentes ayant montré que la squalamine était capable potentialiser les activités de certains antibiotiques tels que la tétracycline [17]. Il serait souhaitable de déterminer la potentialité des dérivés aminostéroïdiens associés à divers antibiotiques afin de renforcer les activités antibactériennes de ces derniers notamment sur les bactéries Gram négative multirésistantes.

Par ailleurs, la squalamine et les dérivés bis(polyaminostéroïdiens) pourraient être utilisés sous forme d'aérosol dans le traitement d'infections pulmonaires. Le

traitement par inhalation cible spécifiquement les poumons permettant ainsi de réduire ainsi les effets systémiques secondaires. Un travail a été entrepris dans notre laboratoire pour déterminer l'activité *in vivo* des aérosols de dérivés aminosteroïdiens sur un modèle animal. Enfin, la squalamine et les dérivés bis(polyaminosteroïdiens) peuvent constituer une alternative pour combattre les bactéries pathogènes multirésistantes qui sont responsables d'infections nosocomiales.

ANNEXE

Article 6

Synergistic activity of sulbactam combined with colistin against colistin-resistant *Acinetobacter baumannii*

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To the Editor:

Acinetobacter baumannii is an emerging multidrug resistant (MDR) pathogen that is responsible for community and hospital-acquired infections that are difficult to control and to treat [1]. With the increasing antimicrobial resistance to carbapenems, colistin is often the treatment of last resort, but colistin-resistant clinical isolates have already been reported. Since therapeutic options are very limited or null in some cases of infections with pan-drug resistant bacteria there is an urgent need to find new antibiotic strategies. In this study, we report the in vitro synergistic activity of a combination of colistin with sulbactam against colistin-resistant *A. baumannii* strains.

We have recently reported the clinical case from a French patient with a bloodstream infection with a MDR but colistin-susceptible *A. baumannii* strain. After a 4-week treatment with colistin, a strain resistant to this antibiotic was isolated from the sputum of the patient [2]. The two strains were resistant to all other antibiotics commonly tested including the extended-spectrum cephalosporins, aztreonam, fluoroquinolones, aminoglycosides, tigecycline and carbapenems (imipenem minimum inhibitory concentrations [MIC] 16mg/L for both strains). The MICs of colistin and sulbactam were determined using the E test method (AB Biodisk, Solna, Sweden) and interpreted according to guidelines recommended by the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (www.sfm-microbiologie.org/) The first *A. baumannii* strain isolated

showed a colistin MIC of 0.064 mg/L and a sulbactam MIC of 2 mg/L, and the second one (Fig. 1,A), a colistin MIC of 32 mg/L but remained sensitive to sulbactam, with a MIC of 2 mg/L. In order to investigate a potential synergy of colistin with sulbactam on the colistin-resistant strain, we have performed a test consisting of a combination of E-test strips that were placed on Mueller–Hinton agar medium, with a 90° angle at the intersection between the scales at the respective minimal inhibitory concentrations (MICs), and the plates were incubated at 37°C for 24 h. The MICs were interpreted at the point of intersection between the inhibition zone and the E-test strip. Results showed a synergistic activity of colistin plus sulbactam since colistin MIC decreased from 32 to 4 mg/L and sulbactam MIC decreased from 2 to 0.5 mg/L (Fig. 1,B).

In the case of this French patient, a colistin-resistant *A. baumannii* strain has emerged in vivo following the use of colistin as single therapy [2]. A strong association between the use of this antibiotic and emergence of resistance in clinical strains of *A. baumannii* has been reported recently in Spain [3,4]. Therefore, there is a risk of colistin-resistant *A. baumannii* emergence during treatment of colistin as a monotherapy. Colistin acts by modifying the negative charges of the outer membrane of Gram-negative bacteria. The mechanisms of resistance to colistin in *A. baumannii* are not well known and there are so far only two putative mechanisms elucidated in the scientific literature: mutations in two genes that constitute a two-component system (PmrAB) involved in the modification of lipid A, the major

constituent of Lipopolysaccharide membrane and mutations, deletions or insertions in genes essential for synthesis of lipid A (lpxA, lpxC, and lpxD genes) [2]. In order to limit the emergence of such strains due to selection of resistant mutants, synergistic combination with other compounds with both correct in vitro activity and a different mechanism of action are warranted as proposed in our study. Sulbactam is an antibiotic used as a suicide inhibitor of serine beta-lactamases, most of which belong to Ambler class A or C. However, it has been shown that it had also an intrinsic activity against MDR *A. baumannii* strains *in vitro*, suggesting that this compound may be a therapeutic option for cases of infection caused by MDR *A. baumannii* [5]. Moreover, it has been shown that patients infected by *A. baumannii*, including by strains resistant to carbapenems, improved or were cured upon treatment with sulbactam alone or in combination with other antibiotics [1]. Despite the lack of well-controlled clinical studies, our results suggest that sulbactam may be considered as a good option, in association with colistin, in the treatment of MDR *A. baumannii* infections, especially for infections by *A. baumannii* resistant to imipenem.

In conclusion, the increasing use of colistin for treating infections due to MDR *A. baumannii* worldwide will inevitably increase the recovery rate of colistin-resistant isolates in the future. Although the optimal treatment is not currently well established for MDR *A. baumannii* infections, antibiotic combination with sulbactam may provide significant benefit over monotherapy and improve the chance of

survival. We believe that MIC against sulbactam should be systematically determined for carbapenem-resistant *A. baumannii* and added to colistin in the treatment of patients to avoid the emergence of colistin-resistant *A. baumannii* strains as recently exemplified in France and in Spain [2,3].

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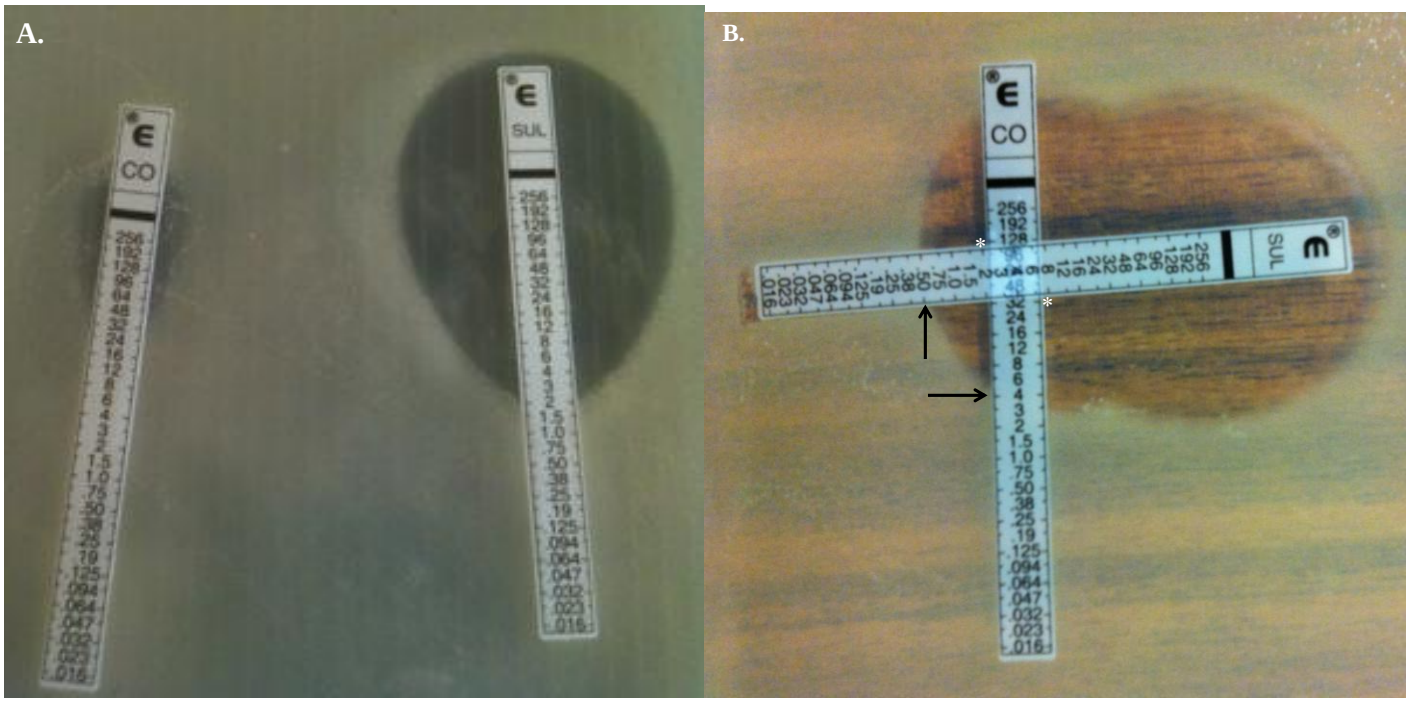
Figure 1.

A. Colistin minimal inhibitory concentration (MIC) and sulbactam MIC of the colistin-resistant *A. baumannii* strain, given by the E-test. Colistin MIC: 32 mg/L; sulbactam MIC: 2 mg/L.

B. Synergy shown by the E-test. Colistin MIC is decreased from 32 to 4, whereas sulbactam MIC is decreased from 2 to 0.5, as indicated by arrows (asterisks denote original MICs).



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